SYMPOSIUM: HYPERLIPIDEMIA

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Chairmen

Hypolipidemic Activity of (——)-Hydroxycitrate

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ABSTRACT

The influence of (−)-hydroxycitrate, a potent competitive inhibitor of adenosine triphosphate (ATP) citrate lyase, on serum triglyceride and cholesterol levels, and in vitro and in vivo rates of hepatic fatty acid and cholesterol synthesis was investigated in normal and hyperlipidemic rat model systems. (−)-Hydroxycitrate reduced equivalently the biosynthesis of triglycerides, phospholipids, cholesterol, diglycerides, cholesteryl esters, and free fatty acids in isolated liver cells. In vivo hepatic rates of fatty acid and cholesterol synthesis determined in meal-fed normolipidemic rats were suppressed significantly by the oral administration of (−)-hydroxycitrate for 6 hr, when control animals exhibited maximal rates of lipid synthesis; serum triglyceride and cholesterol levels were significantly reduced by (−)-hydroxycitrate. In two hypertriglyceridemic models—the genetically obese Zucker rat and the fructose-treated rat—elevated triglyceride levels were due, in part, to enhanced hepatic rates of fatty acid synthesis. (−)-Hydroxycitrate significantly reduced the hypertriglyceridemia and hyperlipogenesis in both models. The marked hypertriglyceridemia exhibited by the triton-treated rat was only minimally due to increased hepatic lipogenesis; (−)-hydroxycitrate significantly inhibited both serum triglyceride levels and lipogenesis in this model.

INTRODUCTION

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proach to hyperlipidemia is defined, in the present context, as intervention in the synthesis, storage, or utilization pathways which maintain serum triglyceride and cholesterol levels. (−)-Hydroxycitrate was an appropriate compound to examine the feasibility of lowering blood lipid levels by metabolic regulation, since it was shown to be a potent compeititve inhibitor (K_i 0.2 μM, K_m citrate 70 μM) of adenosine triphosphate (ATP) citrate lyase (EC 4.1.3.8) (1,2). This extramitochondrial enzyme catalyzes the cleavage of citrate to oxaloacetate and acetyl CoA, and is particularly important in maintaining the acetyl CoA pool during hyperlipogenic conditions, e.g., a nutritional state of high carbohydrate feeding (3,4).

Previous investigations have demonstrated (−)-hydroxycitrate to be an effective inhibitor of fatty acid (5-12) and cholesterol synthesis (6,13,14) in vitro and in vivo in the tissues which serve as sources of newly synthesized lipid, i.e. liver (5-9,11), adipose tissue (7,10), small intestine (7), and skin (12). Of the four stereoisomers of hydroxycitrate, only the (−)-isomer exhibited this activity; the other three isomers and citrate were inactive (6). Some of the carbohydrate carbons and electrons diverted from lipid biosynthesis by (−)-hydroxycitrate were apparently channeled into hepatic glycogen, since significant increases in both glycogen content and the conversion of radiolabeled isotopes into glycogen were observed (8). The acute (8) and chronic (15,16) oral administration of a nontoxic dose of (−)-hydroxycitrate significantly reduced appetite in both lean and obese rats and mice. This (−)-hydroxycitrate-induced anorexia produced a reduction in body weight gain which was reflected in significant decreases in totoal body lipid (15,16); protein levels were unchanged. When food intake was restricted to that consumed by the (−)-hydroxycitrate-treated rats, equivalent reductions in weight gain and total body lipid were observed in the (−)-hydroxycitrate-treated and pair-fed groups (15). However, normal rates of hepatic lipogenesis were observed in the pair-fed rats, whereas the (−)-hydroxycitrate group demonstrated significantly reduced rates of fatty acid synthesis (7).

The present study was designed to examine the effect of (−)-hydroxycitrate on hepatic lipid synthesis and serum lipid levels in normolipidemic and three hypertriglyceridemic models (the genetically obese Zucker rat, the fructose rat, and the triton rat). Various characteristics of these hypterlipidemic models suggest their usefulness in evaluating potential lipid lowering agents. Increased levels of serum triglycerides and cholesterol (17-19), phospholipids and free fatty acids (20) were reported in the Zucker obese rat, which carries a mutation for obesity inherited as an autosomal Mendelian recessive trait (21). Although hyperphagic, the hypertriglyceridemia persisted even when the obese animals were restricted to a caloric intake equivalent to lean controls (22). Recent experiments suggested that an overproduction of very low density lipoprotein (VLDL) rather than an underutilization of these lipoproteins accounted for the hypertriglyceridemia in the obese rats (20). Additionally, an enhanced catabolism of VLDL occurred in the obese, suggesting a compensatory response to the increased secretion rates of VLDL (23).

Fructose administration was reported to produce hepatic hyperlipogenesis (24-27) and hypertriglyceridemia (28,29). Elevated hepatic lipogenic enzyme activities were demonstrated after fructose administration (for review, see ref. 30).

Triton WR-1339, a nonionic surface active agent produced a marked hypertriglyceridemia and hypercholesterolemia after parenteral administration (31,32). Increased cholesterogenesis and enhanced activity of HMG CoA reductase accounted, apparently, for the hypercholesterolemia (33,34). The hypertriglyceridemia appeared to be due to decreased utilization of the very low density lipoporteins, caused by a triton-mediated physical alteration of the lipoproteins rendering them refractive to the lipolytic enzymes in blood and tissues (35,36).

EXPERIMENTAL PROCEDURES

Animals, Diets, and Treatment

Female rats [either Charles River CD strain (2 months old), Charles River Breeding Laboratories, Wilmington, MA., or genetically obese and lean Zucker (4 months old), Harriet G. Bird Memorial Laboratory, Stow, MA] were individually housed in wire-bottomed cages in a temperature-regulated (22 C) light-controlled room (12 hr light, 6 a.m. to 6 p.m., and 12 hr dark, 6 p.m. to 6 a.m.). They had free access to a commercial diet (Purina Rodent Chow, Ralston Purina Co., St. Louis, MO) and water for at least 1 wk prior to experimental alimentation. All animals were fasted 48 hr, then meal-fed the G-70 diet from 8 to 11 a.m. daily (Charles River rats), or ad libitum fed the G-70 diet (Zucker rats) for periods indicated below. The G-70 diet consisted of 70% glucose, 23% vitamin-free casein, 5% PH salt mixture IV (37). 1% vitamin mixture (24), 1% corn oil, 0.05% BHT, and 40 g/kg cellulose. Our previous in-

vestigations demonstrated that this regimen induced elevated in vivo hepatic rates of fatty acid and cholesterol synthesis in the rat (7,24).

In the Zucker rat experiment, animals were given two daily oral doses of (−)-hydroxycitrate (1.32 mmoles/kg/5 ml) or H_2O by intubation at 8 a.m. and 4 p.m. for 7 to 13 days. On the experimental day, in vivo rates of hepatic fatty acid synthesis and serum triglycerides were determined 4½ hr after the a.m. dose of (−)-hydroxycitrate.

In the fructose experiment, rats were meal-fed the G-70 diet for 7 days then given a drinking tube containing either H_2O or a 10% fructose solution for 28 hr. Rats were administered (−)-hydroxycitrate (1.32 mmoles/kg/5 ml t.i.d.) or H_2O orally by intubation at 3 intervals during this 28 hr period: 1 hr before the meal, 5 hr after the end of the meal, and on the next day 1 hr before the meal. In vivo rates of hepatic fatty acid synthesis and serum triglycerides were determined at hr 28.

In the triton experiment, rats were meal-fed the G-70 diet for 7 days, then administered i.v. triton WR 1339 (250 mg/kg/1.25 ml) in 0.14 M NaCl or 0.14 M NaCl alone, and followed immediately by an oral dose of (−)-hydroxycitrate (2.63 mmoles/kg/5 ml) or H_2O by intubation. In vivo rates of hepatic fatty acid synthesis and serum triglycerides were determined 6 hr after the initiation of the meal.

Preparation of Isolated Hepatocytes and Determination of In Vitro Rates of Lipid Synthesis

Meal-fed Charles River rats were anesthetized with Nembutal (62.5 mg/kg i.p.), livers were perfused in situ and hepatocytes isolated as described previously (38,39) with the following modifications: (a) the perfusion media contained 16.5 mM glucose; (b) the liver was not incubated after perfusion; and (c) the liver was minced in cold Krebs-Henseleit bicarbonate buffer pH 7.4, following the perfusion. The hepatocytes were isolated after washing four times in Krebs-Henseleit buffer. Cell integrity was determined using trypan blue; 95% of the cells excluded the dye. All incubations were carried out in a total volume of 2.1 ml consisting of: 1 ml cells (containing between 10-20 mg cells by dry wt); 1 ml Krebs-Henseleit buffer pH 7.4; 50 μl radioactive material (1 μCi [U-^{14}C]alanine, 1 mCi ^3H_2O, 1 μmole alanine); 25 μl 25.2% glucose; and 25 μl of the appropriate concentration of (−)-hydroxycitrate. Each flask was gassed for 15 sec with 95% O_2-5% CO_2 and stoppered during the incubation. The reaction flasks were incubated in a gyrotory water bath at 37 C for 60 min.

For determination of lipogenesis and cholesterogenesis, the reaction was stopped by the addition of 2.1 ml of 5 N NaOH; the flask contents were transferred to tubes, rinsed with two 1 ml water rinses, and saponified overnight at 90 C. Following saponification, the slurry was extracted three times with 5 ml petroleum ether, the extract washed with 7.5 ml water, evaporated to dryness, and the sterols precipitated by a modified Sperry and Webb procedure (40). The evaporated extract was dissolved in 10 ml of acetone:ethanol (1:1), acidified with 0.1 ml of 10% acetic acid, and 1 mg cold cholesterol in 0.1 ml acetone added to facilitate precipitation. The mixture was precipitated with 4 ml of 0.5% digitonin in 50% ethanol, and the precipitate washed with 15 ml of acetone:ether (1:2), then with 15 ml of ether. The precipitate was dissolved in 1 ml of methanol and radioactivity determined in a liquid scintillation spectrometer using a toluene PPO-POPOP scintillation mixture. Fatty acids were isolated as follows: the aqueous saponified phase was acidified with 2.5 ml of 5 N HCl, extracted three times with 5 ml petroleum ether, evaporated to dryness and radioactivity determined as described above. Data are expressed as nmoles ^3H_2O or [^{14}C]alanine converted into fatty acids or cholesterol per mg dry wt cells per 60 min.

For the determination of the rate of synthesis of other lipid classes, the reaction was stopped by the addition of 6 ml methanol: chloroform (2:1), and lipids were extracted by a modification of the Bligh and Dyer procedure (41). The chloroform extract was washed three times with 50% methanol to remove ^3H_2O, then evaporated to dryness and resolubilized in chloroform:methanol (1:1). Aliquots were spotted on ITLC-SG paper and run in isooctane:isopropyl acetate (100:3) (42). The origin containing phospholipids was cut off and counted as previously described (43). The chromatogram was placed in an iodine chamber until the lipids were visible; spots were marked and the chromatogram placed in air until the iodine disappeared. Areas containing cholesterol, cholesteryl esters, triglycerides, free fatty acids, and diglycerides were cut and placed in scintillation vials with a toluene PPO-POPOP scintillation mixture, and radioactivity was determined in a liquid scintillation spectrometer.

Determination of In Vivo Rates of Lipogenesis and Cholesterogenesis

Rates of synthesis were determined 30 min after the intravenous injection of a radioactive pulse consisting of 1 mCi ^3H_2O, 5 μCi [U-

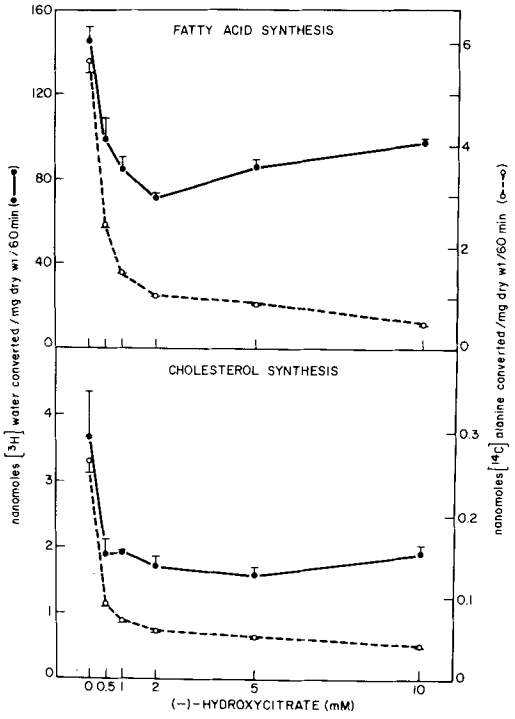

FIG. 1. Effect of (−)-hydroxycitrate concentration on the rates of fatty acid and cholesterol synthesis in isolated rat hepatocytes. Each flask contained 8.7 mg cells by dry wt. Cells were incubated as described in Experimental Procedures. Rates were determined from ^3H_2O (0—0) and [^{14}C]alanine(0- - -0). Each point represents the mean of 4 determinations and the bar indicate the S.E.

^{14}C]alanine, 12.3 mg alanine, and 30.6 mg α-ketoglutarate (as an amine acceptor for transaminase) in 0.25 ml saline solution. Animals were killed by decapitation. Livers were immediately excised, and 2 g samples saponified overnight at 70 C in 5 volumes of 10% alcoholic potassium hydroxide. Cholesterol synthesis was measured as follows: saponified samples were extracted three times with 5 ml petroleum ether and washed with 7.5 ml H_2O to obtain sterols which were determined by a modified Sperry and Webb procedure (40). The evaporated extract was treated as described above for isolated hepatocytes with the following exceptions: no cold cholesterol was added and the final precipitate was disolved in 2 ml methanol before counting. For the determination of fatty acid synthesis, aqueous saponified samples were acidified and extracted, and radioactivity determined as previously described (7). Data are expressed as μmoles ^3H_2O or nmoles [^{14}C]-alanine converted to cholesterol or fatty acids per g liver per 30 min.

Determination of Serum Triglycerides and Cholesterol

Following decapitation, blood was collected, stored on ice for 30 min, and centrifuged. The serum was assayed for triglycerides by a fluorometric method (44) and for cholesterol by an enzymatic procedure (45).

Sources of Chemicals

The dietary components were supplied by Nutritional Biochemicals, Cleveland, OH; the BHT by Hoffmann-La Roche Inc., Nutley, NJ; the ^3H_2O and [^{14}C]alanine by New England Nuclear, Boston, MA; the Triton WR 1339 by Ruger Chemical Co., Irvington, NJ; the cholesterol test kit by Abbott Labs., Pasadena, CA; ITLC-SG by Gelman Instrument Co., Ann Arbor, MI; isooctane by Burdick and Jackson, Muskegon, MI; and all other chemicals by Sigma, St. Louis, MO. (−)-Hydroxycitrate trisodium salt was synthesized by Guthrie and Kierstead of the Chemical Division of Hoffmann-La Roche Inc.

Statistical Analysis

Date were processed statistically for outliers (46). A two-sided "t" test was used to evaluate all experimental results (47).

RESULTS AND DISCUSSION

Effect of (−)-Hydroxycitrate on Lipid Biosynthesis in Isolated Rat Hepatocytes

Fatty acid and cholesterol synthesis was inhibited significantly from ^3H_2O and [^{14}C]-alanine at all concentrations of (−)-hydroxycitrate employed (0.5 mM to 20 mM) (Fig. 1). ^3H_2O was utilized to determine the total rate of lipogenesis and cholesterogenesis since it was incorporated into these lipids independent of the source of carbon precursors of acetyl CoA (48,49). [^{14}C]alanine was employed as an acetyl CoA precursor. Two mM (−)-hydroxycitrate produced near maximum inhibition of the conversion of ^3H_2O and [^{14}C]alanine to these lipids, equivalent to 50% and 80%, respectively. The greater effectiveness of (−)-hydroxycitrate in suppressing fatty acid and cholesterol synthesis from [^{14}C]alanine than ^3H_2O was analogous to that observed in the intact rat (Fig. 2 and 3) and suggested that other sources of extramitochondrial acetyl CoA which were not labeled by [^{14}C]alanine were being utilized for lipid synthesis, thus diluting the specific activity of the [^{14}C]acetyl CoA pool derived from [^{14}C]alanine. Half maximum inhibition of fatty acid and cholesterol synthesis in isolated liver cells occurred at ca. 0.3 to 0.4 mM (−)-hydroxycitrate (Fig. 1),

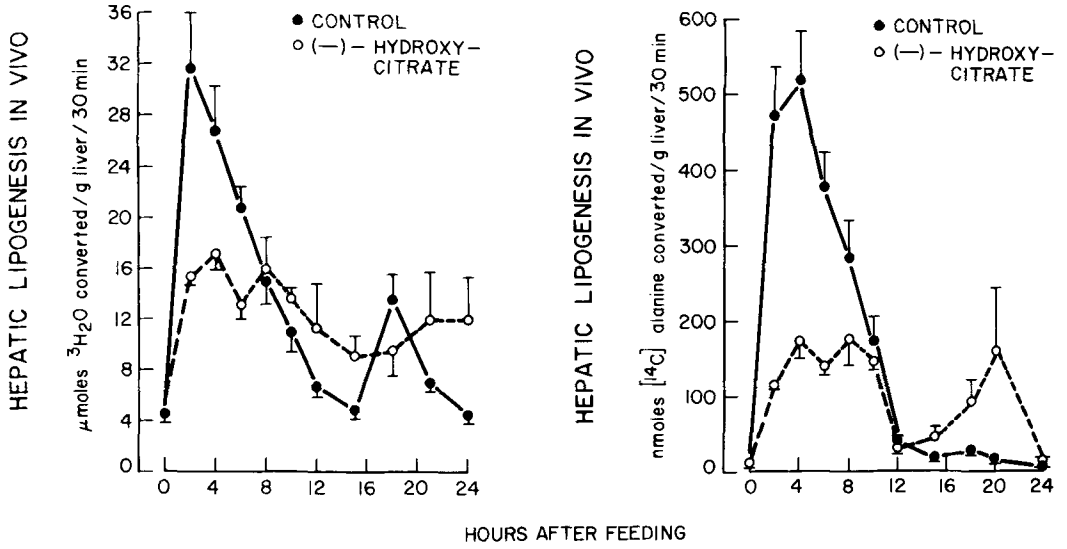

FIG. 2. Effect of the oral administration of (–)-hydroxycitrate in pair-fed rats on the in vivo rate of hepatic lipogenesis determined over a 24 hr period. Rats were meal-fed for 6 days and on the 7th day given orally by intubation either H_2O or (–)-hydroxycitrate (2.63 mmoles/kg) immediately before being given 8.7 g of food. In vivo rates of lipogenesis were determined 30 min after the i.v. administration of the radioactive pulse (given immediately after the 3 hr meal). Each point represents the mean of 8 values. The bars represent the S.E. at each point.

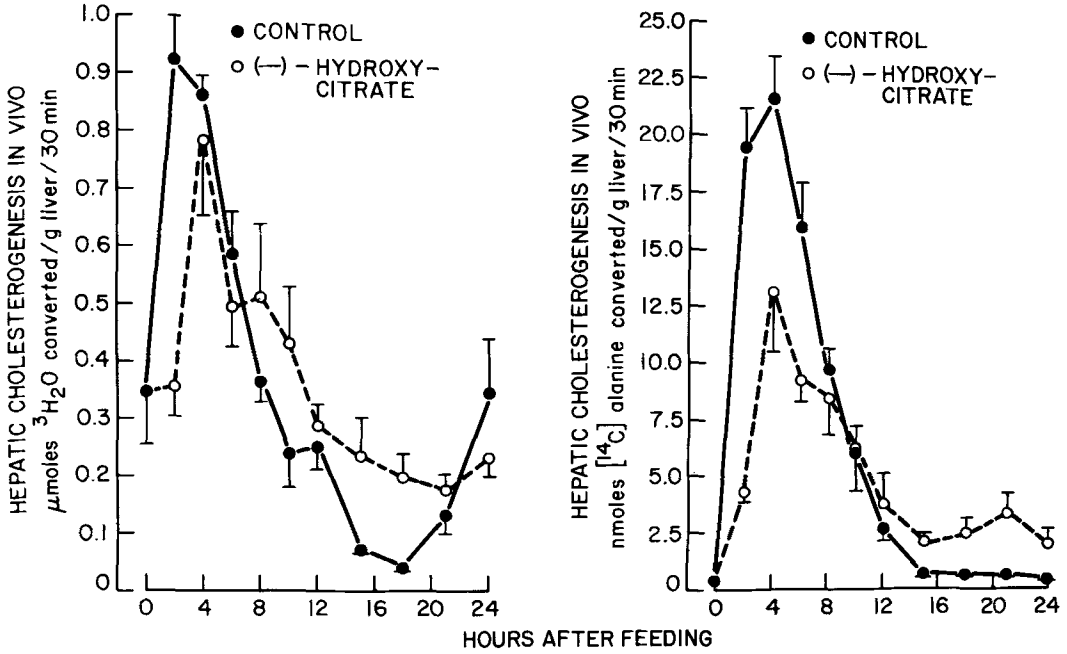

FIG. 3. Effect of the oral administration of (–)-hydroxycitrate in pair-fed rats on the in vivo rate of hepatic cholesterogenesis determined over a 24 hr period. Rats were meal-fed for 6 days and on the 7th day given orally by intubation either H_2O or (–)-hydroxycitrate (2.63 mmoles/kg) immediately before being given 8.7 g of food. In vivo rates of cholesterogenesis were determined 30 min after the i.v. administration of the radioactive pulse (given immediately after the 3 hr meal). Each point represents the mean of 8 values. The bars represent the S.E. at each point.

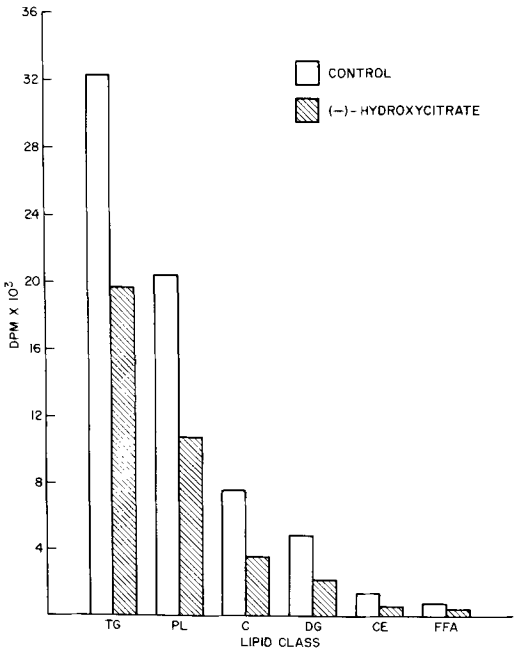

FIG. 4. Effect of (−)-hydroxycitrate (2 mM) on the distribution of lipids synthesized in isolated rat hepatocytes. Each flask contained 12.2 mg dry weight of cells. Cells were incubated and extracted as described in Experimental Procedures. The lipid classes are: TG (triglycerides), PL (phospholipids), C (cholesterol), DG (diglycerides), CE (cholesteryl esters), and FFA (free fatty acids). Each bar represents the mean of six determinations. Data are expressed as DPM [^{14}C]alanine converted into each lipid class. Similar results were obtained for the incorporation of $^{3}H_2O$ into the same lipid classes.

which was similar to the approximate K_i observed for in vivo fatty acid synthesis after the i.p. administration of (−)-hydroxycitrate (6). This is 15- to 20-fold higher than the reported K_i for the purified ATP citrate lyase (1) and the K_i for [^{14}C]citrate (1 mM) incorporation into fatty acids in a 105,000 x g rat liver supernatant system (data not shown). These data suggested that the transport of (−)-hydroxycitrate into the intact liver cell was limited and recent experiments (data not shown) demonstrated an uptake of (−)-hydroxycitrate into isolated rat hepatocytes of ca. 5%.

The effect of 2 mM (−)-hydroxycitrate on the synthesis of various lipid classes in isolated liver cells was examined (Fig. 4). The relative rates of [^{14}C]alanine conversion to lipids in the control flasks were as follows (percent of total lipid synthesized is indicated in parentheses): triglycerides (48%) > phospholipids (31%) > cholesterol (11%) > diglycerides (7%) > cholesteryl esters (2%) and free fatty acids (1%). (−)-Hydroxycitrate significantly inhibited the synthesis of all lipid classes to a similar extent (percent inhibition is indicated in parentheses): triglycerides (39%), phospholipids (47%), cholesterol (52%), diglycerides (54%), cholesteryl esters (58%), and free fatty acids (40%).

Effect of (−)-Hydroxycitrate on Hepatic Rates of Fatty Acid and Cholesterol Synthesis In Vivo

The influence of (−)-hydroxycitrate on hepatic fatty acid and cholesterol synthesis in vivo over a 24 hr period was investigated in a meal-fed rat model (Fig. 2 and 3). Controls pair-fed to (−)-hydroxycitrate-treated rats were employed to eliminate any differences in lipid synthesis due to changes in caloric intake, since previous investigations demonstrated (−)-hydroxycitrate to be an effective appetite suppressant (8,15,16). Rats were administered (−)-hydroxycitrate (2.63 mmoles/kg) orally by intubation immediately before the 3-hr meal. At 2, 4, 6, 8, 10, 12, 15, 18, 21 and 24 hr after the initiation of the meal, rates of fatty acid and cholesterol synthesis were determined. Control rats demonstrated maximum synthesis of both lipids from $^{3}H_2O$ and [^{14}C]alanine 2 to 4 hr after the initiation of the meal; rates of fatty acid and cholesterol synthesis returned to prefed levels ca. 12 hr after feeding and remained low until refeeding. Cholesterol was synthesized at ca. 3 to 4% of the rate of fatty acid synthesis.

(−)-Hydroxycitrate administration significantly decreased the lipogenic rates determined from $^{3}H_2O$ and [^{14}C]alanine to fatty acids for 0 to 6 hr after refeeding. During the 12 to 24 hr period when control rats demonstrated low rates of fatty acid synthesis, the (−)-hydroxycitrate-treated animals exhibited slightly enhanced lipogenic rates. These increases in the rates of conversion of $^{3}H_2O$ and [^{14}C]alanine into fatty acids were statistically significant at hr 15 and hr 15, 18 and 24, respectively.

(−)-Hydroxycitrate inhibited cholesterol synthesis to a lesser extent than fatty acid synthesis. Rates of conversion of $^{3}H_2O$ were significantly less than controls for 2 hr after feeding; [^{14}C]alanine conversion was significantly depressed in the (−)-hydroxycitrate rats for 6 hr after initiation of the meal. As observed with lipogenesis, (−)-hydroxycitrate-treated animals demonstrated mildly enhanced rates of cholesterogensis when control rates were low. The conversion of $^{3}H_2O$ and [^{14}C]-analine to cholesterol was significantly increased in the (−)-hydroxycitrate animals at hr 15 and 18, and hr 15, 18, and 21, respectively. This apparent increased flow of carbons and

TABLE I

Effect of (−)-Hydroxycitrate on Serum Lipid Levels in Normolipidemic Rats

	Serum lipids			
	Triglycerides		Cholesterol	
Treatment[a]	mg%[b]	% of control	mg%[b]	% of control
Control	85 ± 5	100	41 ± 4	100
Citrate	98 ± 6	131	45 ± 3	110
(−)-Hydroxycitrate	69 ± 5[c]	81	29 ± 2[d]	71

[a]Three groups of seven rats each were fasted 48 hr then fed the G-70 diet for 5 days from 8 to 11 a.m. On the experimental day, rats were dosed orally by intubation 1 hr before the meal with either water or 2.63 mmoles/kg citrate or (−)-hydroxycitrate. Serum was collected 1/2 hr after the end of the 3 hr meal following decapitation.

[b]Each value represents the mean ± S.E.

[c]$p<0.05$

[d]$p<0.01$.

electrons into fatty acids and cholesterol in (−)-hydroxycitrate-treated rats during periods when controls demonstrated fasting biosynthetic rates has been consistently observed, although its significance was questioned (7). The magnitude of this increased flux appeared to be insufficient to compensate for the carbohydrate carbons and electrons which were diverted from lipid synthesis by (−)-hydroxycitrate. However, since previous studies indicated that (−)-hydroxycitrate-treated rats lost an amount of body fat similar to that of pair-fed controls (15), the possibility existed that the total lipogenic capacity over a 24 hr period of the (−)-hydroxycitrate-treated rats was equivalent to controls. Previous investigations demonstrated that some of these diverted carbons and electrons were apparently channeled into hepatic glycogen since significant increases in both glycogen content and the conversion of radio-labeled isotopes into glycogen were observed (8). These increases were apparent from 4 to 10 hr after the initiation of the meal. From 12 to 24 hr, the rates of glycogenesis and hepatic glycogen levels were indistinguishable in control and treated animals. The apparent enhanced rates of lipogenesis and cholesterogenesis from 12 to 24 hr reported here may reflect the utilization of these increased glycogen stores.

Effect of (−)-Hydroxycitrate on Serum Lipid Levels in Normolipidemic and Hyperlipidemic Rat Models

Serum triglyceride and cholesterol levels were determined 4½ hr after the oral administration of (−)-hydroxycitrate or citrate to meal-fed rats (Table I). (−)-Hydroxycitrate (2.63 mmoles/kg) significantly reduced circulating levels of triglycerides and cholesterol by 19% and 29%, respectively. Equimolar doses of citrate did not significantly alter the concentration of these blood lipids.

The ability of (−)-hydroxycitrate to reduce serum triglyceride levels was examined in 3 hypertriglyceridemic rat model systems: the genetically obese Zucker rat, the fructose rat, and the triton rat (Table II). In vivo hepatic rates of fatty acid synthesis and serum triglyceride levels were determined simultanesouly.

Zucker lean and obese rats were fed ad libitum and administered (−)-hydroxycitrate (1.32 mmoles/kg b.i.d.) orally by intubation. Control obese rats demonstrated significantly elevated rates of fatty acid synthesis from both ^3H_2O (460% of lean rate) and [^{14}C]alanine (1140% of lean rate); these enhanced rates were correlated with significantly higher levels of serum triglycerides (350% of lean level). (−)-Hydroxycitrate reduced significantly the lipogenic rates and circulating triglyceride levels in the lean and obese rats. Hepatic rates of conversion of ^3H_2O to fatty acids in lean and obese rats treated with (−)-hydroxycitrate were reduced to 79% and 53% of the control rate, respectively. Lipogenesis from [^{14}C]alanine in lean and obese (−)-hydroxycitrate-treated rats was decreased to 77% and 37% of controls, respectively. (−)-Hydroxycitrate reduced serum triglyceride concentrations in lean and obese animals to 73% and 65% of the control levels, respectively.

Fructose administered in the drinking water as a 10% solution for 28 hr increased hepatic rates of fatty acid synthesis from both precursors by ca. 1.4-fold and enhanced circulating triglyceride levels by 2-fold. (−)-Hydroxycitrate given orally by intubation at 1.32 mmoles/kg t.i.d. (hr 0, 9, and 24) significantly inhibited lipogenic rates from both precursors. In control and fructose-treated rats, the conversion of ^3H_2O was reduced by 55% and 36%, respec-

TABLE II

Effect of (−)-Hydroxycitrate on Hepatic Fatty Acid Synthesis and Serum Triglyceride Levels in Three Hyperlipidemic Rat Models[ab]

Model	Description	(−)-Hydroxycitrate mmoles/kg	Fatty acid synthesis: μmoles ^3H_2O coverted	Fatty acid synthesis: nmoles[^{14}C]alanine coverted	Serum triglycerides mg%
Zucker	Lean	–	13.2 ± 1.6	137 ± 40	103 ± 6
	Lean	1.32 b.i.d.	10.4 ± 1.0	106 ± 43	75 ± 6
	Obese	–	60.5 ± 6.9	1563 ± 43	362 ± 60
	Obese	1.32 b.i.d.	32.2 ± 7.7	575 ± 204	234 ± 82
Fructose	−Fructose	–	24.0 ± 3.1	655 ± 102	59 ± 6
	−Fructose	1.32 t.i.d.	10.9 ± 1.3	189 ± 36	45 ± 5
	+Fructose	–	29.8 ± 2.0	1029 ± 72	116 ± 10
	+Fructose	1.32 t.i.d.	19.2 ± 0.9	619 ± 143	67 ± 4
Triton	−Triton	–	301. ± 2.5	811 ± 108	76 ± 6
	−Triton	1.63	16.1 ± 2.8	259 ± 86	33 ± 1
	+Triton	–	40.4 ± 3.2	998 ± 91	750 ± 5
	+Triton	2.63	22.9 ± 1.8	405 ± 51	516 ± 58

[a]Animals (three to seven per group) were treated as described in the Experimental Procedure section prior to the determination of lipogenesis and serum triglycerides. Data are expressed as μmoles ^3H_2O or nmoles [^{14}C] alanine converted to fatty acids per g liver per 30 min and mg% serum triglycerides. Each value represents the mean ± S.E.

[b](−)-Hydroxycitrate significantly reduced fatty acid synthesis from ^3H_2O and [^{14}C]alanine in the following groups: obese Zucker, -fructose, +fructose, -triton, and +triton rats. (−)-Hydroxycitrate significantly reduced serum triglycerides in the lean Zucker, +fructose, -triton. and +triton rats.

tively; [^{14}C]alanine rates were depressed by 71% and 40%, respectively. These changes were reflected in reduced serum triglyceride levels, which were suppressed in control and fructose rats by 24% and 42%, respectively. The (−)-hydroxycitrate-mediated suppression of circulating triglyceride levels was not due to reduced food intake, since the hypotriglyceridemic activity of (−)-hydroxycitrate was observed in both fed and fasted rats of the fructose model (50).

Triton WR-1339 induced a 10-fold increase in circulating triglyceride levels, but fatty acid synthesis was elevated only ca. 30%, when these parameters were measured 6 hr after triton administration. (−)-Hydroxycitrate (2.63 mmoles/kg) given orally by intubation significantly reduced lipogenic rates and serum triglyceride levels. The conversion of ^3H_2O in control and triton-treated rats was depressed by 47% and 43%, respectively, by (−)-hydroxycitrate; [^{14}C]alanine conversion was reduced by 68% and 59%, respectively. (−)-Hydroxycitrate treatment decreased triglyceride levels in control and triton rats by 57% and 31%, respectively. These data confirm and extend a previous investigation, which demonstrated that genetically and experimentally induced hypertriglyceridemia was correlated with hyperlipogenesis, and that inhibiting fatty acid synthesis by (−)-hydroxycitrate administration reduced serum triglyceride levels (50).

In conclusion, the studies described here suggest that hyperlipidemia may be therapeutically managed by metabolic regulation, since (−)-hydroxycitrate inhibited hepatic lipid synthesis and reduced circulating triglyceride and cholesterol levels. The enhanced rates of lipogenesis demonstrated in three hypertriglyceridemic models–the genetically obese Zucker rat, the fructose rat, and the triton rat–further support this treatment approach of employing an inhibitor of lipid synthesis.

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Effects of Colestipol Hydrochloride and Neomycin Sulfate on Cholesterol Turnover in the Rat

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ABSTRACT

Three groups of male rats were fed diets containing the bile acid sequestrant colestipol hydrochloride (1%), neomycin sulfate (0.25%), or basic diet during the test. After 15 days, each rat was injected IV with 3.9 μCi cholesterol-1,2-^{3}H complexed with serum lipoproteins; specific radioactivity of the total serum cholesterol was measured at several time intervals for a period of 7 weeks. Computer analysis of the data indicated that the turnover of cholesterol could best be fitted by a three-pool model. In pool 1, colestipol HCl caused a significant increase in production rate (10.09 to 15.96 mg/day) and the excretion rate constant (0.53 to 0.79 day^{-1}) of cholesterol without significantly altering the size of the pool or serum cholesterol concentrations. These results are compatible with an agent capable of binding bile acids in the rat but do not cause a decrease of the sterol pool because of an adequate compensatory increase in cholesterol biosynthesis. Neomycin SO_4 caused a significant reduction in serum cholesterol (9%) without altering turnover parameters and apparently exerts its hypocholesterolemia by some mechanism other than bile acid sequestration.

INTRODUCTION

Colestipol HCl (Colestid®, The Upjohn Company, Kalamazoo, Michigan.) is a high molecular weight anion exchange polymer, prepared by reacting polyethylenepolyamine and 1-chloro-2,3-epoxypropane, and contains ca. one of five amine nitrogens protonated as the chloride salt. Oral administration of the bile acid-binding colestipol increases the fecal excretion of bile acids by reducing their intestinal reabsorption, thereby reducing cholesterol levels in dogs and cockerels (1,2) and in humans (3-5). However, in normocholesterolemic rats fed colestipol HCl, serum cholesterol levels are unchanged from control due to increased cholesterol biosynthesis (6). Neomycin SO_4, a polybasic antibiotic, reduces both serum cholesterol (7,8) and the exchangeable pool of cholesterol in man only (9), probably by increasing fecal excretion of bile acids and neutral sterols (10,11). The administration of the antibiotic in a basic or cholesterol rich diet to rats did not significantly alter serum or liver cholesterol levels from respective controls (12). The primary purpose of the present study was to utilize the method of pulse labeling with isotopic cholesterol to determine the distribution and turnover of exchangeable cholesterol in rats fed colestipol HCl or neomycin SO_4; results may aid in determining the mode of action of these agents. Some of the findings have been published previously as an abstract (13).

METHODS

Three groups of Upjohn:TUC(SD)spf male rats, 15 per group, with an initial fasted weight of 324 g (range 310-347 g) were used in this study. The control and treated groups received a basic diet (14) with the treated receiving supplements in the diet of 1% colestipol HCl (Lot #1CX24) and 0.25% neomycin SO_4 (Lot #1JV12 code 249550). The rats were permitted water and their respective diets ad libitum during the test. After 15 days, each rat was injected IV with 0.43 ml rat serum containing 3.9 μCi of cholesterol-1,2-^{3}H. Blood samples (0.3 or 0.5 ml) were taken without anesthesia from the jugular vein at various time intervals for 7 wk (15). On the 50th day, rats were anesthetized with Cyclopal® (sodium cyclopentenylallybarbiturate), drained of blood, and prepared for further analyses.

The cholesterol-1,2-^{3}H (New England Nuclear, Boston, MA; specific activity 50 Ci/mM) was found, by thin layer chromatography on silica gel and scanning with a radiochromatogram scanner (Model 885, Vanguard Instrument Corp., LaGrange, IL), to be radiochemically pure. To 48 ml of serum from fasted rats, 480 μl (ca. 1 μl = 1 μCi) of an alcoholic solution of the labeled cholesterol was added and shaken gently for 16 hr at 37 C. The serum was sterilized by passage through a Nalgene® Filter Unit (Nalge Sybron Corp., Rochester, NY) with a pore size of 0.2 μ.

Cholesterol concentration and radioassay were accomplished after serum samples were saponified and extracted according to the method of Abell et al. (16). An aliquot of the

hexane was taken to dryness in a counting vial and, after the addition of scintillation fluid containing 0.4% 2,5-diphenyloxazole (PPO) and 0.005% p-bis[2-(5-phenyloxazolyl)]-benzene (POPOP) in toluene, was counted in a liquid scintillation spectrometer (Model 3375, Packard Instrument Co., Downers Grove, IL). Serum cholesterol concentrations were determined by gas chromatography of hexane aliquots using 5α-cholestane as internal standard. Samples were chromatographed on a glass column (18 x 1/4 inch O.D.) packed with 3.8% UCW98 on 60-80 mesh diaport S (Hewlett-Packard) in a Hewlett-Packard model 400 gas chromatograph with an oven temperature of 210 C.

On the 50th day, anesthetized rats were drained of blood and perfused with 0.9% saline through the abdominal aorta. Good blanching of the tissues resulted; the gastrointestinal tract was washed to remove feces and returned to the carcass. The brain and spinal cord were removed and weighed in separate flasks. To each weighed carcass, 1 volume of saturated aqueous KOH containing 0.75 g KOH per g tissue and 1/2 volume of ethanol and 1 ml benzene solution of radiochemically pure cholesterol-4-^{14}C (New England Nuclear, Boston, MA) containing 118700 dpm was added to monitor the extraction of cholesterol. The carcass was digested in a water-bath at 37 C overnight and completely dissolved, leaving only a few bone fragments. A 10 ml aliquot was removed; ethanol concentration was adjusted to 50% and extracted three times with 35 ml of hexane. Cholesterol assays were performed as described previously using a 6′ x 1/4 in. O.D. glass column packed with OV-17 on 80-100 mesh gaschrom Q (Applied Science Labs, State College, PA) with 5β-cholestan-3β-ol as internal standard (oven temperature 245 C). Tritium and carbon-14 were measured simultaneously (17). Tritium was measured in the low energy red channel while ^{14}C was measured in the high energy green channel. The absolute radioactivities for the isotopes were determined by the use of simultaneous equations; quenching was determined using the automatic external standard and prepared efficiency correlation curves.

The central nervous system (CNS) for each rat was saponified with alcoholic KOH to which 1 ml cholesterol-4-^{14}C in ethanol containing 1187 dpm was added. Final concentration was 3 ml per g of CNS in a 25% solution of KOH in 95% ethanol. Digestion and extraction of the tissues were made according to the method of Duncan and Best (18). Aliquots of hexane were used for radioassay by the double labeling technique; cholesterol concentrations were determined using the conditions described for serum analyses.

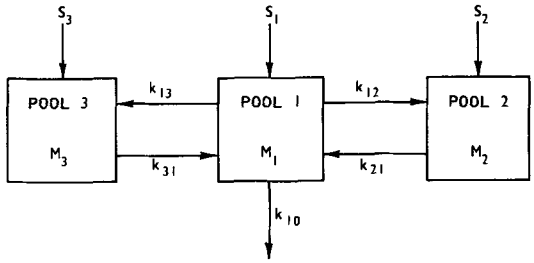

FIG. 1. Three-pool model. Rate constants indicated by k values and sizes of pools denoted by M values. The addition of exogenous and endogenous cholesterol to various pools indicated by S values. Cholesterol is excreted primarily from Pool 1, and $k_{10}XM_1$ (PR_1) represents total body cholesterol turnover.

The analyses of the data using the program NONLIN (19), a nonlinear estimation program, and the IBM-370 computer indicated that the turnover in rats can best be described by a three-pool model (Fig. 1). The specific activity function for the first pool is described by the equation:

$$C(t) = Q_1e^{-\alpha t} + Q_2e^{-\beta t} + Q_3e^{-\gamma t} \quad (I)$$

where,

$$Q_1 = \frac{D}{V}\left[\frac{\alpha^2 - (K_{21} + K_{31})\alpha + K_{21}K_{31}}{(\beta - \alpha)(\gamma - \alpha)}\right]$$

$$Q_2 = \frac{D}{V}\left[\frac{\beta^2 - (K_{21} + K_{31})\beta + K_{21}K_{31}}{(\alpha - \beta)(\gamma - \beta)}\right]$$

$$Q_3 = \frac{D}{V}\left[\frac{\gamma^2 - (K_{21} + K_{31})\gamma + K_{21}K_{31}}{(\alpha - \gamma)(\beta - \gamma)}\right]$$

D, V, and K_{ij} are dose (dpm), volume of pool 1 (mg) and rate constant for transfer from pool i to pool j, respectively. α, β, and γ are the solutions to the equation: $S^3 + (K_{1\cdot} + K_{21} + K_{31})S^2 + (K_{1\cdot}K_{21} + K_{1\cdot}K_{31} + K_{21}K_{31} - K_{12}K_{21} - K_{13}K_{31})S + K_{10}K_{21}K_{31} = 0$ where $K_{1\cdot} = K_{10} + K_{12} + K_{13}$ is the sum of the rate constants originating from pool 1.

From equation 1 we can compute the parameters:

Production rate in pool 1 = $K_{10}V$ and

$$\text{Area } (t = 0 \text{ to } \infty) = \frac{D}{V}\left[\frac{Q_1}{\alpha} + \frac{Q_2}{\beta} + \frac{Q_3}{\gamma}\right]$$

Data were transformed by W(t) = 1/C(t) in order to estimate the turnover parameters with maximum precision.

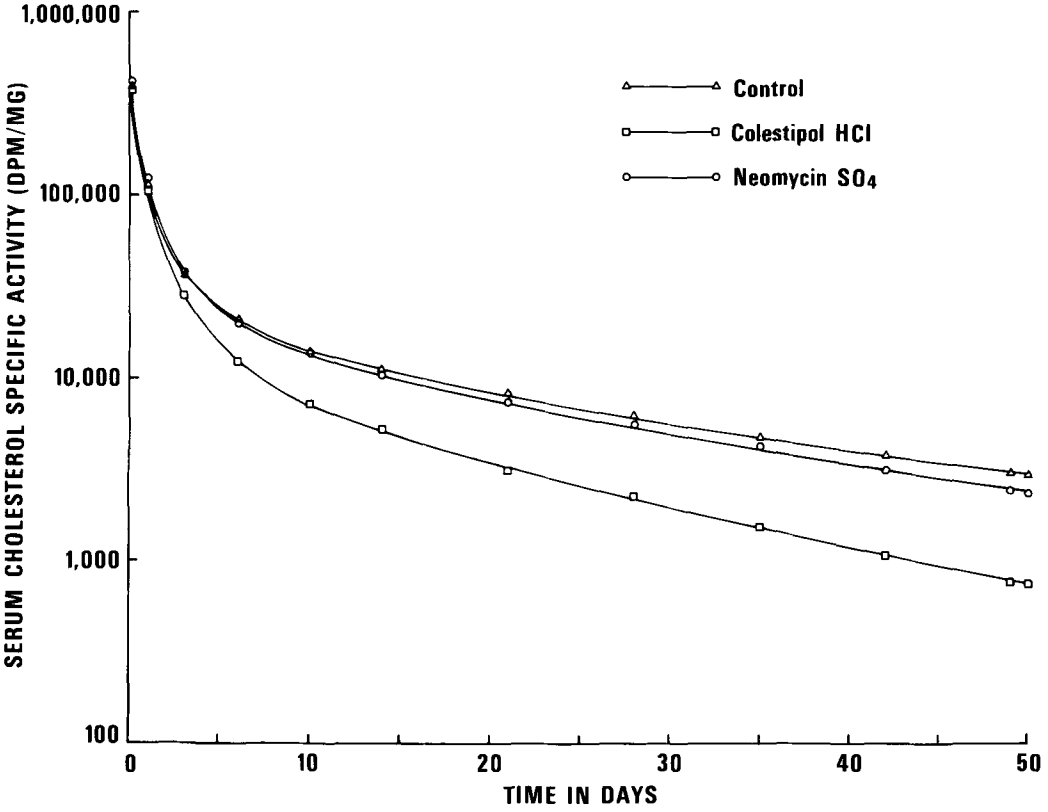

FIG. 2. Semilogarithmic plots of serum cholesterol specific activity versus time after I.V. administration of 3.9 μCi cholesterol-1,2-^{3}H cholesterol complexed with rat serum lipoporoteins. Each point represents mean of 15 animals.

TABLE I

Turnover Parameters as Determined by Three-Pool Model System[a]

Parameter	Control	Colestipol HCl	Neomycin SO_4
D/V (dpm/mg) x 10^4	45.085 ± 1.770	42.413 ± 1.250	48.497 ± 1.210
K_{12} (day^{-1})	0.543 ± 0.018	0.445[b] ± 0.025	0.470[c] ± 0.021
K_{21} (day^{-1})	0.465 ± 0.019	0.536[c] ± 0.025	0.476 ± 0.014
K_{10} (day^{-1})	0.528 ± 0.017	0.789[b] ± 0.021	0.582 ± 0.015
K_{13} (day^{-1})	0.497 ± 0.0077	0.307[b] ± 0.0079	0.475 ± 0.0104
K_{31} (day^{-1})	0.076 ± 0.0017	0.073 ± 0.0018	0.079 ± 0.0019
M_1 (mg)	19.30 ± 0.667	20.40 ± 0.635	17.76 ± 0.436
PR_1 (mg/day)	10.09 ± 0.273	15.96[b] ± 0.356	10.26 ± 0.144
AREA (0-50) x 10^4	77.212 ± 1.987	52.391[b] ± 1.155	77.407 ± 1.004
AREA (0-∞) x 10^4	85.583 ± 2.404	53.887[b] ± 1.208	83.384 ± 1.192

[a]Mean values ± S.E.M. for 15 animals per group.
[b]Significantly different from control, $P<0.01$
[c]Significantly different from control, $P<0.05$.

Statistical analyses of all cholesterol turnover parameters, serum cholesterol, body weight change, and food intake were made using a one-way classification design. Significance of differences between control and treatment means was determined by Student's t-test using pooled error variance (20).

The method of analyses of curves of specific activity depends upon a steady state condition including unchanging body weight and constant amount of sterol intake (21). The diet used in the test contained a constant amount of sterols

TABLE II

Effects of Colestipol HCl or Neomycin SO_4 on Serum Cholesterol, Body Weight, and Food Intake[a]

Parameter	Control	Colestipol HCl	Neomycin SO_4
Serum cholesterol, mg/100 ml	96.1 ± 1.4	95.9 ± 1.0	87.3[b] ± 0.8
Body weight change, g	76.4 ± 5.6	83.7 ± 4.7	59.1[b] ± 4.2
Food intake, g	897 ± 22.3	948 ± 19.7	842 ± 12.2

[a]Mean values ± S.E.M. for 15 animals per group.
[b]Significantly different from control, $P<0.01$

TABLE III

Carcass Analyses[a]

Group	Cholesterol		
	Observed mg	mg/kg	Specific activity dpm/mg
Control	502 ± 11.2	1068 ± 19.5	2992 ± 134
Colestipol HCl	492 ± 7.5	1028 ± 7.4	1060[b] ± 44
Neomycin SO_4	472[c] ± 8.6	1072 ± 9.5	2566[b] ± 82

[a]Mean values ± S.E.M. for 15 animals per group.
[b]Significantly different from control, $P<0.01$.
[c]Significantly different from control, $P<0.05$.

(9 mg/100 g); average food intake of the three groups did not differ significantly. However, the average weight gain for the three groups was about 18% over the length of the study. This gain in body weight may be included into the model by permitting V in equation I to increase linearly with time with a total increase of 18%. The parameter estimates and fits of the curves did not change appreciably from the values not corrected for weight gain.

RESULTS

The semilogarithmic plots of the mean serum specific activities vs. time for control or treated with colestipol HCl or neomycin SO_4 appears in Fig. 2. Group mean parameters calculated from the turnover data for each individual animal are shown in Table I. In pool 1, colestipol HCl caused a significant increase in production rate (PR_1) and the excretion rate constant (K_{10}) without altering the size of the pool (M_1). Colestipol HCl treatment significantly reduced from control the rate constants (K_{12}, K_{13}), areas 0-50 and 0-∞, and increased rate constant (K_{21}). This model assumes that cholesterol is removed only by way of pool 1 and PR_1 and represents the metabolic turnover of cholesterol from the animal body. The metabolic turnover of cholesterol is identical to production rate (PR_1) of cholesterol when the rate of exit from pool 2 (K_{20}) and pool 3 (K_{30}) equal zero. Other investigations have served to strengthen the concept that the primary exit of cholesterol in the system occurs mainly through pool 1 and peripheral tissues and organs excrete little cholesterol (21,22). Neomycin SO_4 reduced the rate constant (K_{12}); none of the other turnover parameters were considered significantly different from control.

The mean results for serum cholesterol, body weight change, and food intake for treated and control groups are shown in Table II. Colestipol HCl treatment did not significantly alter any of these parameters when compared to control. The unchanged serum cholesterol concentrations caused by colestipol HCl verifies the unaltered pool size (M_1) obtained from the model (Table I). However, neomycin SO_4 treatment reduced significantly serum cholesterol levels (9%) and body weight but had little effect on food intake when compared to control.

The mean carcass analyses for control and treated groups appears in Table III. The total cholesterol of the treated groups, expressed as mg/kg of body weight, did not differ significantly from control. The reductions in carcass cholesterol specific activities by colestipol HCl and neomycin SO_4 in treated animals were significant. The marked reduction of carcass specific activity and increased production rate of cholesterol in the colestipol HCl-treated

TABLE IV

Central Nervous System Analyses[a]

Group	Cholesterol	
	mg/g	Specific activity dpm/mg
Control	21.8 ± 0.34	499 ± 17.7
Colestipol HCl	22.3 ± 0.37	279[b] ± 9.0
Neomycin SO_4	22.3 ± 0.41	479 ± 18.6

[a]Mean values ± S.E.M. for 15 animals per group.
[b]Significantly different from control, $P<0.01$.

animals reflect the loss and dilution of radioactivity due to bile acid sequestration. The rat is known to accelerate the rate of newly synthesized cholesterol, by the liver, to compensate for the loss of bile acids (23). Although neomycin SO_4 reduced carcass specific activity, its mechanism of action as a hypocholesterolemic agent is apparently not by bile acid sequestration.

The results of the central nervous system analyses are shown in Table IV. The concentrations of cholesterol as mg/g of tissue of the treated groups did not vary significantly from control. Only colestipol HCl-treated animals had significantly reduced cholesterol specific activities as was noted in the carcasses of these animals.

DISCUSSION

Colestipol HCl did not affect the cholesterol concentrations of pool 1 or serum cholesterol concentrations, but markedly increased the production rate and rate of removal of cholesterol from pool 1. If we assume that production rate is a valid estimation of metabolic turnover, then colestipol HCl increased by 58% the rate of cholesterol degradation and excretion from the animal body. These results are compatible with an agent capable of binding bile acids in the rat, an animal that can compensate for increased loss of bile acids by markedly increasing cholesterol biosynthesis (6). Recently, Goodman et al. (24) have demonstrated in a small number of human subjects that a three-pool model provides a significantly better description of long-term cholesterol turnover (40 wk) than did a two-pool model. Turnover studies carried out for shorter periods (12-13 wk) are valid when analyzed as a two-pool model if production rate is reduced by 8-9%. The same investigators (24) also showed that colestipol HCl given to human subjects resulted in a significant reduction in serum cholesterol concentration and increased both production rate and turnover rate of pool 1.

On the other hand, neomycin SO_4 significantly reduced serum cholesterol (9%), carcass cholesterol specific activity, and the rate constant K_{12} but did not affect production rate, size of pool 1, and other turnover parameters. Although neomycin SO_4 has been reported to bind bile acids in vitro (25), it caused none of the characteristic changes in turnover parameters seen with the bile acid sequestrant colestipol HCl. Recently, in vivo studies by Thompson et al. (26) have shown that neomycin SO_4 inhibited lymphatic transport of labeled oleic acid and cholesterol but did not inhibit the absorption and biliary re-excretion of taurocholate and taurodeoxycholate. Their results suggested that the basic neomycin precipitates the mixed micellar solutions by ionic interaction with fatty acids and thereby interferes with the absorption of both fats and cholesterol. Furthermore, rats fed cholestyramine, another bile acid sequestrant, increased the hepatic synthesis of cholesterol due to the interruption of enterohepatic circulation of bile acids while neomycin SO_4 did not (27). It is clear from these and our results that neomycin SO_4 does not effect bile acids in the same way as the seuqestrants cholestyramine and colestipol HCl. Sedaghat et al. (28) have reported that neomycin caused hypocholesterolemia in human subjects but did not change the slopes of cholesterol-specific activity time curves. They failed to find any consistent increase in fecal acidic steroids and it appeared that neomycin is not a bile acid sequestrant but possibly exerts its hypocholesterolemic activity by increasing fecal neutral steroid excretion.

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Linoleic Acid Amides: Effect on Cholesteremia and Atherosclerosis

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ABSTRACT

Several of a series of linoleic acid amides have been reported to inhibit cholesterol-induced atherosclerosis in rabbits. The three amides which have been studied to the greatest extent are (in order of increasing activity) N-cyclohexyl linoleamide (AC23), N(α methylbenzyl) linoleamide (AC223), and N[α-phenyl-β-(p-tolyl) ethyl] linoleamide (AC485). We have found AC223 to inhibit cholesterol absorption in rats and to slightly inhibit exogenous but not endogenous cholesteremia in rabbits. In a fiber-free diet, AC223 reduces serum cholesterol and liver triglyceride levels. Rats were also fed a basal semipurified diet with and without AC223. Fecal excretion of labeled exogenous (as [^{14}C] cholesterol) or endogenous (as [^{14}C] mevalonolactone) steroid was 44 and 43% higher in drug treated groups. The mechanism of hypocholesteremic action of the linoleamides appears to involve inhibition of cholesterol absorption.

In 1966, Nakatani et al. (1) reported on a new cholesterol lowering compound, the N-cyclohexylamine amide of linoleic acid (linolexamide, AC-23) (Fig. 1). At a dosage of 0.2% of the diet, this material was found to reduce liver cholesterol levels in rats fed 1% cholesterol. At 12 wk, liver cholesterol levels in rats fed the drug plus cholesterol were 48% those of rats fed cholesterol alone; at 18 wk, the liver cholesterol in the test animals was 49% that of the controls. The compound had no effect on liver cholesterol levels in the absence of dietary cholesterol. Toki and Nakatani (2) demonstrated that the hypocholesteremic property of linolexamide was not due to its linoleic acid content. The effect of linolexamide on cholesterol induced atherosclerosis in rabbits was compared with equal daily levels (400 mg) of linoleic acid, ethyl linoleate, and safflower oil. The levels of atherosclerosis (graded on a 0-4 basis) in those experiments were: control–2.4; linolexamide–0.8; linoleic acid–2.5; ethyl linoleate–3.0; and safflower oil–2.0.

It was further shown (3) that the hypocholesteremic property was not common to all linoleic acid amides. As Table I illustrates, at equivalent dosages, different amides of linoleic acid gave widely disparate results. Thus, the cholesterol-lowering effect was peculiar to specific amides of linoleic acid.

We (4) investigated the effect of various dosages of linolexamide upon atherosclerosis in rabbits fed 2% cholesterol in 6% corn oil. As Table II illustrates, a dose of 600 mg/day was about 40% more effective than one of 300 mg. However, increasing the dose of drug to 900 mg/day did not cause a concomitant increase in effectiveness.

Toki (Table I) had shown that N (α methylbenzyl) linoleamide (AC-223) (Fig. 2) was considerably more effective than linolexamide in lowering serum and liver cholesterol levels in cholesterol-fed rabbits. Further work (5) showed that at comparable doses, AC-223 was considerably more hypocholesteremic and antiatherogenic than was AC-23 (Table III). When fed to rabbits at a level of 200 mg/day, AC-223

TABLE I

Influence of Various Linoleic Acid Amides on Atherosclerosis in Rabbits[a]

Amine[b]	No.	Cholesterol		Atherosclerosis
		Serum, mg/dl	Liver, g/100 g	
---	10	2586	1.76	1.88
Cyclohexyl	10	746	0.85	0.75
N-isopropyl	4	1808	1.89	1.67
N,N-diphenyl	4	1500	0.90	0.90
N,N-methylcyclohexyl	6	1578	0.99	1.22
N (α methylbenzyl)	6	81	0.32	0.17

[a]After Toki and Nakatani (3).

[b]All amides fed at 400 mg/day.

TABLE II

Influence of Various Dosages of N-Cyclohexyl Linoleamide on Atherosclerosis in Rabbits
(All diets contain 2% cholesterol and 6% corn oil: 2 month study)

Dosage[a]	No.	Wt. gain (g)	Liver wt. (g)	Cholesterol		Atherosclerosis	
				Serum, mg/dl	Liver, g/100 g	Arch	Thoracic
Low	59/70	317	107.7	1423	5.74	1.68	1.18
Medium	22/24	279	96.8	1115	3.71	1.10	0.66
High	20/24	272	90.0	1074	4.30	0.96	0.80
Control	62/70	190	113.2	1915	7.98	1.93	0.88

[a]Low – 300 mg/day; medium – 600 mg/day; high – 900 mg/day.

TABLE III

Influence of Linoleamides on Experimental Atherosclerosis in Rabbits[a]

Drug	No.	Dose (mg)	Cholesterol		Atherosclerosis
			Serum, mg/dl	Liver, g/100 g	
Control	7	-	1688 ± 148	2.82 ± 0.48	2.57 ± 0.43
AC223	5	100	476 ± 223	0.64 ± 0.11	1.20 ± 0.58
AC223	6	200	285 ± 58	0.73 ± 0.25	0.83 ± 0.31
AC223	6	400	61 ± 16	0.30 ± 0.02	0.33 ± 0.21
AC23	6	200	1051 ± 279	1.03 ± 0.20	1.33 ± 0.42
AC23	6	400	429 ± 152	0.51 ± 0.12	0.83 ± 0.48

[a]After Toki et al. (2).

TABLE IV

Influence of Optical Isomers of N (α Methylbenzyl) Linoleamide on Atherosclerosis in Rabbits[a]

Isomer	No.	Dose (mg)	Serum cholesterol mg/dl	Atherosclerosis
Control	9	-	1999 ± 199	1.89 ± 0.26
DL	8	25	1204 ± 232	1.38 ± 0.32
DL	9	50	1128 ± 262	1.44 ± 0.44
D	9	25	746 ± 220	0.89 ± 0.26
D	8	50	647 ± 101	1.25 ± 0.45
L	9	25	1501 ± 282	1.44 ± 0.34
L	9	50	1472 ± 184	2.22 ± 0.32

[a]After Fukushima and Nakatani (6).

resulted in serum and liver cholesterol levels that were, respectively, 73 and 29% lower than those observed when AC-23 was fed. At a dose of 400 mg/day, AC-223 normalized serum and liver cholesterol levels. Since N (α methylbenzyl) linoleamide is optically active, it was deemed interesting to ascertain if one stereoisomer was more active than the other. Fukushima and Nakatani (6) investigated the activity of the isomeric forms of AC-223. They showed (Table IV) that the D isomer was considerably more hypocholesteremic than the racemic compound, and reduced atherosclerosis in rabbits by ca. 40%.

In the course of continuing research (7), it was found that N [α phenyl-β-(p-tolyl)ethyl] linoleamide (AC-485) (Fig. 3) was even more effective than AC-223 in lowering serum and liver cholesterol levels in cholesterol-fed rabbits. Thus, when AC-485 at a dosage of 5 mg/day was compared with AC-223 at a dose of 25 mg, it gave serum cholesterol levels that were 20% lower, liver cholesterol levels that were 24% lower, and atherosclerosis that was 30% less severe (Table V).

Nagata et al. (8) have studied the effects of AC-485 on cholesterol absorption. One 15 mg dose of drug was followed by the administration of labeled cholesterol or cholesteryl oleate. Absorption of either free or esterified cholesterol was significantly inhibited, with the latter being inhibited to a greater extent. The results

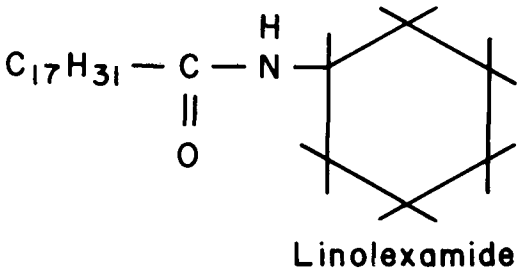

FIG. 1

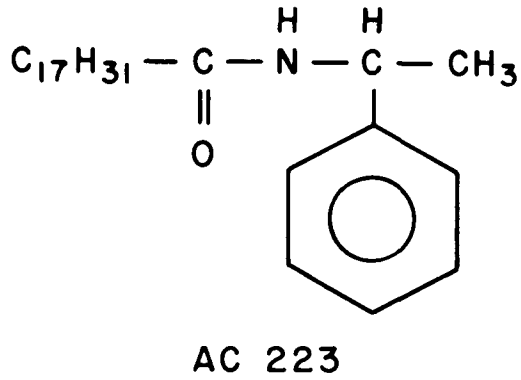

FIG. 2

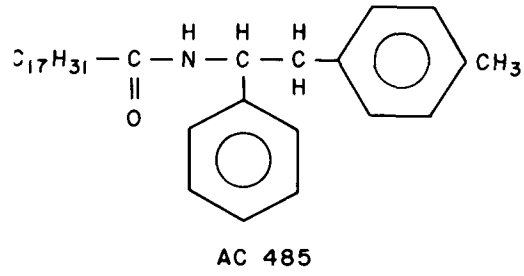

FIG. 3

suggest an effect of the drug on hydrolysis of cholesteryl esters. Studies of the effects of AC-485 on intestinal cholesterol esterase activity showed that the drug enhanced esterification and inhibited hydrolysis.

In cholesterol-fed rats, Nagata et al. (9) showed that AC-485 fed at a level of 0.02% of the diet was as effective in inhibiting absorption as 1% sitosterol. The effect was more marked in male than in female rats.

The two more effective linoleamides (AC-223 and AC-485) have been subjected to clinical test. Ono (10) administered AC-223 to 28 patients with various lipid abnormalities. The dose was 1.5 g/day administered in divided doses three times per day (500 mg after each meal). Over a one year period, there was a 19% reduction in serum cholesterol ($p<0.01$) and a 10% drop in neutral lipids. Phospholipid levels remained unchanged. There was no evidence of harmful side effects.

Takeuchi and Yamamura (11) fed AC-485 (150 or 300 mg) to men who had deliberately elevated their cholesterol levels by eating 9 egg yolks per day for 2 wk, and after returning to normal levels, repeated this dietary regimen together with drug treatment. It is of passing interest that when young men (18-20 yr) were fed egg yolks, their average serum cholesterol levels rose from 188 to 205 mg/dl ($p<0.02$), whereas in older men (40-45 yr) the rise was from 195 to 229 mg/dl ($p<0.01$). While the cholesterol levels of the two groups were not significantly different prior to the egg yolk diets, they were significantly different ($p<0.02$) afterwards. It was found that ingestion of AC-485 together with egg yolks prevented the rise in serum cholesterol levels (Table VI).

Although the original paper by Nakatani et al. (1) had suggested that the linoleamides affected cholesterol absorption, more data were required. Our experiments (12) showed that when N-cyclohexyl linoleamide was fed as 0.3%

TABLE V

Influence of Linoleamides on Experimental Atherosclerosis in Rabbits[a]

Drug	No.	Dose (mg)	Cholesterol		Avg. Atherosclerosis
			Serum, mg/dl	Liver, g/100 g	
Control	11	-	1797 ± 159	2.96 ± 0.37	2.36 ± 0.28
AC223	7	25	1200 ± 284	1.57 ± 0.23[c]	1.43 ± 0.53
AC223	7	50	726 ± 216[b]	1.28 ± 0.27[b]	1.29 ± 0.57
AC485	13	5	956 ± 147[b]	1.20 ± 0.14[b]	1.00 ± 0.25[c]
AC485	14	10	636 ± 119[b]	0.88 ± 0.10[b]	0.57 ± 0.14[b]
AC485	7	25	341 ± 97[b]	0.62 ± 0.10[b]	0.14 ± 0.14[b]

[a]After Nakatani et al. (7).

[b]$p<0.01$ vs. control.

[c]$p<0.05$ vs. control.

TABLE VI

Influence of AC485 on Serum Cholesterol Levels of Nine Men Eating Nine Egg Yolks Daily[a]

Egg yolk (EY)		EY + 300 mg drug		EY + 150 mg drug	
Pre-	Post	Pre-	Post	Pre-	Post
185.0 ± 5.2	222.6 ± 8.1[b]	188.3 ± 6.8	179.8 ± 6.0	175.8 ± 7.0	183.0 ± 10.8

[a]After Takeuchi and Yamamura (11).
[b]$p<0.01$.

TABLE VII

Influence of DL-N (α Methylbenzyl) Linoleamide on Cholesterol Absorption in the Rat (Compound fed 21 days at 0.3%)

	Recovery (dpm x 10^4)			
	[7α-^{3}H] cholesterol		[4-^{14}C] cholesterol	
	Test	Control	Test	Control
Serum cholesterol	1.77 ± 0.12	5.22 ± 1.22	0.80 ± 0.07	0.96 ± 0.10
Liver cholesterol	4.91 ± 0.38	5.71 ± 1.70	0.24 ± 0.05	0.30 ± 0.03
Serum plus liver (% dose)	3.04 ± 0.19[a]	4.97 ± 0.79	1.04 ± 0.03[b]	1.26 ± 0.09

[a]vs. control $p<0.01$.
[b]vs. control $p<0.05$.

TABLE VIII

Effect of N (α Methylbenzyl) Linoleamide (AC223) on Endogenous and Exogenous Lipemia in Rabbits (1 month feeding)

		Wt. gain	Liver		Serum, mg/dl		Liver, g/100 g	
Group[a]	No.	(g)	Wt. (g)	% body wt.	Cholesterol	Triglyceride	Cholesterol	Triglyceride
SP	7/7	388	51.7	3.35 ± 0.32	229 ± 27	42.4 ± 7.8	0.49 ± 0.06	0.77 ± 0.17
SPD	5/7	95	48.5	3.89 ± 0.38	244 ± 63	55.4 ± 10.3	0.59 ± 0.09	0.83 ± 0.20
C	3/7	435	92.0	5.26 ± 0.63	2133 ± 715	52.3 ± 22.3	2.40 ± 0.30	0.28 ± 0.03
CD	7/7	254	79.7	5.03 ± 0.91	1956 ± 276	69.4 ± 10.7	2.04 ± 0.14	0.42 ± 0.09

[a]SP – semipurified diet: 25% casein, 40% sucrose, 14% hydrogenated coconut oil; SPD – SP + AC223 (50 mg/day); C – 2% cholesterol + 6% corn oil; CD – C + AC223 (50 mg/day).

of the diet to rats for 21 days, it had a slight hepatomegalic effect, but did not influence serum or liver cholesterol levels. This drug had a slight, but not significant, influence upon the oxidation of [26-^{14}C]cholesterol to $^{14}CO_2$ by suitably fortified preparations of rat liver mitochondria (16.0 ± 6.2% oxidation by test animal preparations and 11.2 ± 3.7% by controls). When added to rat liver slice preparations (10^{-3} to 5.5 x 10^{-3}M), AC-23 did not affect cholesterol synthesis from sodium [1-^{14}C]acetate (13).

In testing N (α methylbenzyl) linoleamide for its effects in experimental atherosclerosis, Fukushima et al. (14) compared it with an equal dose (400 mg) of β-sitosterol, a well established inhibitor of cholesterol absorption (15). Sitosterol should be fed at two to three times the level of dietary cholesterol for maximum effect (16); at 400 mg it was ineffective, whereas AC-223 reduced serum and liver cholesterol levels to the normal range in rabbits fed 1.6 g of cholesterol daily, and atherosclerosis was virtually eliminated.

Fukushima et al. (17) fed AC-223 (50 mg) together with [^{14}C]cholesterol to rats. They found the compound inhibited cholesterol absorption, as measured by recovery of label from serum and liver, and esterification by ca. 50%. The recovery of [^{3}H]cholesterol from lymph was inhibited by AC-223 but cholesterol esterification was not affected.

We fed the D and L isomers of AC-223 and the racemate to rats for 21 days. The various compounds were added to a cholesterol-free, semipurified diet (18). We noted a slight hepatomegalic effect but no influence on serum or liver cholesterol levels or upon hepatic cho-

TABLE IX

Influence of AC223 (0.3%) on Serum and Liver Lipids of Rats Fed a Fiber Free Diet[a]

Diet	Fiber free (FF)		FF + 1% cholesterol	
AC223	-	+	-	+
Survival	6/6	6/6	6/6	6/6
Weight gain, g	120 ± 8[b]	121 ± 5	93 ± 8	104 ± 9
Liver wt., g[c]	14.0 ± 0.8$_w$	11.6 ± 0.7$_w$	10.7 ± 0.9	10.7 ± 1.1
% body wt.	4.32 ± 0.27$_x$	3.54 ± 0.15$_x$	3.58 ± 0.28	3.49 ± 0.25
Cholesterol				
Serum, mg/dl	208 ± 6.5$_y$	170 ± 6.6$_y$	194 ± 11	196 ± 8
Liver, mg/100 g	378 ± 52	296 ± 22	1613 ± 117	1284 ± 155
Triglycerides				
Serum, mg/dl	100 ± 20	100 ± 34	46 ± 6	50 ± 13
Liver, mg/100 g	2961 ± 646$_z$	856 ± 132$_z$	2573 ± 918	660 ± 49

[a]Sucrose, 60; casein, 24; corn oil, 10; Salt Mix XIV, 5: vitamin mix, 1.

[b]Standard error.

[c]Values bearing same subscript are significantly different.

TABLE X

Influence of AC223 on Endogenous and Exogenous Cholesterol Absorption

	Basal diet[a]		Basal diet + 0.3% AC223	
Survival	12/12		11/12	
Weight gain, g	48 ± 7		60 ± 7	
Liver wt., g[b]	6.8 ± 0.2$_x$		8.3 ± 0.5$_x$	
% body wt.	2.64 ± 0.04$_y$		3.07 ± 0.11$_y$	
Cholesterol, serum mg/dl	165 ± 3		175 ± 9	
liver, mg/100 g	239 ± 9		239 ± 10	
Triglycerides, serum mg/dl	27.2 ± 2.1		29.9 ± 2.6	
liver, mg/100 g	455 ± 41$_z$		613 ± 37$_z$	
Radioactivity recovery[c]	Cholesterol	MVA[d]	Cholesterol	MVA
Serum cholesterol, dpm x 10^3	9.54 ± 0.92	11.31 ± 1.13	8.68 ± 1.07	10.98 ± 1.92
Liver cholesterol, dpm x 10^4	2.92 ± 0.40	4.01 ± 0.15	4.67 ± 0.40	4.68 ± 0.76
Fecal steroids, dpm x 10^5				
Neutral	3.24 ± 0.53	0.98 ± 0.14	5.23 ± 1.12	1.36 ± 0.24
Acidic	1.83 ± 0.41	3.75 ± 0.78	2.08 ± 0.17	5.40 ± 0.84

[a]Mixed cereal, 70; wheat germ, 7; skim milk powder, 22; vitamins, 1.

[b]Values bearing same subscript are significantly different.

[c]Each rat given either 0.5 μCi of [4-^{14}C] cholesterol or 2.0 μCi of [2-^{14}C] mevalonolactone. 4 days before autopsy. Fecal data derived from pooled 4 day collection.

[d]MVA = mevalonolactone.

lesterol synthesis or degradation. When absorption of either [^{3}H]- or [^{14}C]cholesterol was studied, we observed that AC-223 inhibited absorption by 39% when [7 α-^{3}H]cholesterol was used and by 21% when the substrate was [4-^{14}C]cholesterol (Table VII). The difference is due to animal variation and not to the isotope used.

We also tested the effect of AC-223 in rabbits fed a semipurified, cholesterol-free, hypercholesteremic diet, which can be regarded as a model for endogenous hypercholesteremia (19). These animals were compared with others fed a diet containing 2% cholesterol. Both series of diets were fed for 30 days. As Table VIII shows, AC-223, fed at a dosage of 50 mg/day did not affect either serum or liver cholesterol (20) or triglyceride (21) levels in the rabbits fed the semipurified diet. In the cholesterol-fed rabbits, however, serum cholesterol levels were reduced by 8% and liver cholesterol levels were 15% lower. We did not compare levels of atherosclerosis since the semipurified diet must be fed for about 4 months before any athero-

genic effects are discernable.

We have tested the effect of AC-223 on cholesterol levels in rats fed a fiber-free diet of the type used by Ershoff (22) containing 1% cholesterol. The diet (60, sucrose; 24, casein; 10, corn oil; 5, salt mix; 1, vitamins) was fed for 3 wk. In the absence of cholesterol, the drug (0.3% of diet) caused a significant reduction in serum cholesterol as well as a slight drop (22%) in liver cholesterol. Serum triglycerides were unaffected, but liver triglycerides were reduced. In the cholesterol-fed rats, AC-223 did not affect serum lipids, but reduced liver lipids (Table IX).

To further test the effects of AC-223 on the absorption of endogenous and exogenous cholesterol, two groups of 12 rats were fed a diet consisting of cereal, skim milk powder, wheat germ, and vitamins (23) with and without 0.3% of the drug. After 3 wk, half of each group was given an oral dose (0.5 μCi) of [4-^{14}C] cholesterol in propylene glycol and the other half was injected intraperitoneally with an aqueous solution of mevalonolactone (2.0 μCi). After 4 days, the rats were killed, serum and liver lipids and cholesterol radioactivity were determined as well as the neutral and acidic radioactivity in the 4-day pool of feces from each rat. The data are summarized in Table X.

The drug was found to be hepatomegalic. Absorption of exogenous cholesterol was inhibited by AC-223; thus the amount of cholesterol radioactivity in the serum of rats given an oral dose of [4-^{14}C] cholesterol was 9% lower than in control rats, and liver radioactivity was 60% higher. Fecal excretion of neutral steroid radioactivity was 61% higher than in drug-treated rats and acid steroid radioactivity was 14% higher. The effect of AC-223 on endogenous cholesterol (synthesized from intraperitoneally injected [2-^{14}C] mevalonolactone) absorption was similar to its effect on exogenous cholesterol. The radioactivity of total serum and liver cholesterol of the treated rats was 3 and 17% lower, respectively, than in control rats. Excretion of labeled neutral steroids was 39% higher in the rats fed AC-223 and excretion of labeled acid steroids 44% higher.

The data obtained by us and by others all indicate that certain amides of linoleic acid are hypocholesteremic and antiatherogenic and suggest that the mechanism of action of these compounds involves the inhibition of cholesterol absorption.

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Response to the Hypobetalipoproteinemic Agent Adamantyloxyphenyl Piperidine in Hyperlipemic Rats

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ABSTRACT

The hypobetalipoproteinemic activity of U-41,792 (1-[p-(1-adamantyloxy)-phenyl]-piperidine) is a marked and selective reduction of heparin precipitating lipoproteins (low density plus very low density lipoproteins) in cholesterol-cholic acid induced hypercholesterolemic rats. This activity consists of both a reduction in heparin precipitating lipoproteins (HPL) and an increase in high density lipoproteins that are not precipitated by heparin. The increase in high density lipoproteins is routinely noted by decreases in HPL/cholesterol ratios. The pattern of response following single 100 mg/kg doses of U-41,792 was determined. After an I.V. dose was administered in a cottonseed oil emulsion, serum cholesterol levels were reduced, beginning at 8 hr after administration and persisting for 96 hr. Similar results, though delayed somewhat, were obtained after a single oral dose. Activity was accompanied by increases in weight and cholesterol content of livers. After multiple, daily, oral doses, liver weights, total lipids, and cholesterol contents were reduced. Hypobetalipoproteinemic activity was enhanced by prolonged treatments as demonstrated by analyses of serum obtained weekly throughout 7 wk.

INTRODUCTION

Excessive serum lower density lipoproteins (LDL and VLDL) are generally regarded as pathogenic factors in the development of atherosclerosis. High density lipoproteins (HDL) are considered to be nonatherogenic. We developed rapid, inexpensive screening procedures to detect pharmacological agents that decrease lower density or increase high density lipoproteins, or both (1). A compound, U-41,792, 1[p-(1-adamantyloxy)phenyl]-piperidine causes a dramatic reduction in lower density lipoproteins. We designate this activity as hypobetalipoproteinemia. This agent has been active in hypercholesterolemic rats, mice, quail, pigeons, and gerbils (2).

In this report, we examine the effects of single and multiple doses of U-41,792 upon serum and liver lipids of hypercholesterolemic rats. In single dose studies, the rapidity of onset and persistence of action were determined. Multiple-dose studies were used to determine animal drug tolerance, effects upon serum lipoprotein fractions, and effects upon liver weights and lipids.

METHODS

General

Animals: Male UPJ:TUC(SD)spf rats were fed a semipurified diet containing 1.5% cholesterol and 0.5% cholic acid (1). In single dose studies, weanling rats (50 ± 10 g) were fed the diet for 3 days before distribution by weight into groups. These rats were group caged and received diet ad libitum until fasted. In multiple dose studies, the rats were caged singly after distribution into groups. At the end of each study, the rats were weighed, anesthetized with Cyclopal® sodium (80 mg/kg I.P.), and bled into test tubes from severed throats. After bleeding, livers were removed, blotted, weighed, and frozen in small plastic bags until analyzed. In studies where livers were perfused with cold saline before removal, about 5 ml saline per g liver was injected into each liver via the abdominal vena cava.

Drug dosages: In most studies, the drug was dispersed in 0.25% aqueous methyl cellulose vehicle by use of a glass tissue grinder or a blender. Drug concentrations were adjusted to provide the dosage with P.O. administration of 1 ml per 50 ± 2.5 g rat for weanlings and 1 ml per 100 ± 5 g when the rats weighed 100 g or more. When U-41,792 was administered as an emulsion, the drug was dissolved in cottonseed oil, and a 15% fat emulsion containing 5 mg drug per ml was prepared (3). Autoclaving conditions were 15 min at 15 psi (121 C).

Analyses: Individual serum samples were analyzed for total cholesterol by the method of Block et al. (4) and for heparin precipitating lipoproteins (HPL) by the method of Schurr et al. (1). Livers were macerated in their plastic containers. Then 1 g aliquots were dried for solids determination, and 0.5 g aliquots were extracted and washed with aqueous $MgCl_2$ according to Folch et al. (5). Aliquots of the

TABLE I

Hypobetalipoproteinemic Activity 18 Hours After One Oral Dose of U-41,792 Emulsion in Hypercholesterolemic Rats

	No. rats	Oral dose (mg/kg)	Serum Cholesterol (mg/dl)	Serum HPL[a] ($A_{680}X10^3$)	Serum HPL/ Cholesterol	Liver weight[b]
Neg. control	12	-	783[c]	957[c]	1.22[c]	
Control emulsion	12	-	461	710	1.54	4.51
U-41,792 emulsion[d]	4	100	120[c]	135[c]	1.12[c]	5.44[c]
	4	50	168[c]	190[c]	1.13[c]	5.13[c]
	4	25	498	688	1.38	4.72
% Standard deviation			27.3	33.2	11.3	5.6

[a]HPL = heparin precipitating lipoproteins.

[b]As % body weight.

[c]Significantly different from control mean, $P \leq .05$.

[d]Autoclaved emulsion.

Folch extract were dried and taken up in isopropanol for cholesterol determination. Total lipid concentration was determined from a 10 ml aliquot dried to constant weight.

Data were statistically analyzed as a one-way classification (6). All values were transformed to logarithms to achieve more homogenous within-group variances. The mean response for each treatment was compared with the mean observed in the control animals with the least significant difference (LSD) test (7).

Specific

Evaluation of U-41,792 emulsion: Intravenous 15% cottonseed oil emulsion containing U-41,792 was tested to determine if the drug was stable to emulsion preparation and active intravenously. Eighteen groups of hypercholesterolemic weanling rats (four animals per group) were used. Nine groups were injected via a tail vein with 1 ml emulsion per 50 ± 2.5 g. Activities of autoclaved and nonautoclaved U-41,792 emulsion were compared. Another six groups received equal volumes of emulsion via stomach tube and the three remaining groups, as negative controls, received nothing. Cottonseed oil emulsion without drug was used for control groups and to dilute U-41,792 emulsion for drug doses less than 100 mg/kg. The rat groups were fasted and bled 18 hr after treatment.

One dose time-activity responses: To compare the activities of single oral vs. single I.V. doses of U-41,792 with time after dosing, 42 groups of six hypercholesterolemic weanlings per group were used. For each post-treatment time, a control group received emulsion minus drug both I.V. and orally, whereas the other two groups received either U-41,792 emulsion I.V. and control emulsion orally, or vice versa. The emulsion doses were 1 ml per 50 ± 2.5 g of body weight. All groups were dosed within 12 hr. Groups bled 2 to 16 hr posttreatment were fasted for 12 hr, the other groups were bled after a 24 hr fast. Livers were perfused with saline before removal.

Site of cholesterol accumulation: From preliminary studies, we knew that U-26,719 (p-(1-adamantyloxy)-aniline) was equally hypocholesterolemic 16 hr after one or four daily doses. To determine where this cholesterol accumulated, we determined the cholesterol content of four tissues 16 hr after one oral dose of 50 mg/kg. Five controls and five treated rats were used. After bleeding the anesthetized animals, the tissues were removed, cleaned of extraneous tissues, blotted, and weighed. The epididymal fat pads, adrenals, spleen, and a 0.5 g portion of liver from each rat were analyzed. Tissue samples were macerated in isopropyl alcohol with a glass tissue grinder and allowed to extract overnight at room temperature.

Multiple dose effects on serum: To determine whether rats develop a tolerance to U-41,792, four groups of ten rats were fed the diet and dosed orally each day for 7 wk with 0, 6, 25, and 100 mg/kg/day U-41,792 in aqueous vehicle. They were bled serially every 7 days following an 18 hr fast. About 1 ml of blood was taken from the external jugular vein, without anesthesia, as described by Phillips et al. (8).

Multiple dose effects on livers: To determine the effects of multiple doses of U-41,792 on liver weights and lipids, 16 groups of six weanling rats per group were started on the diet and drug treatment simultaneously. After each

TABLE II

Hypobetalipoproteinemic Activity 18 Hours After One Intravenous Dose of U-41,792 in Hypercholesterolemic Rats

	No. rats	IV dose (mg/kg)	Serum Cholesterol (mg/dl)	Serum HPL[a] ($A_{680}X10^3$)	Serum HPL/cholesterol ratio	Liver weight[b]
Control emulsion IV	12	-	637	988	1.55	4.90
		Autoclaved U-41,792 emulsion				
U-41,792 IV	4	100	176[c]	183[c]	1.04[c]	4.53
	4	50	353[c]	562[c]	1.59	5.67[c]
	4	25	407[c]	649[c]	1.60	4.76
		Nonautoclaved U-41,792 emulsion				
U-41,792 IV	4	100	118[c]	130[c]	1.10[c]	5.19[c]
	4	50	300[c]	435[c]	1.45	4.29
	4	25	545	727	1.33	4.50
% Standard deviation			24.9	27.7	12.0	9.5

[a]HPL = heparin precipitating lipoprotein.

[b]As % body weight.

[c]Significantly different from control mean, $P \leq .05$.

experimental period (1, 2, 4, and 7 wk), four groups of rats which received either 0, 50, 100, or 200 mg/kg/day U-41,792 in aqueous vehicle were fasted 18 hr and then bled. After bleeding, the livers were perfused with saline, blotted, weighed, and analyzed for solids, cholesterol, and total lipid content.

Multiple dose effects on lipoproteins: To demonstrate quantitative effects of U-41,792 on lipoprotein concentrations, the individual sera of rats treated for 1 wk, from the above study, were used. The β- and pre-β-lipoproteins were precipitated in a manner similar to our automated procedure (1). During mixing, 0.5 ml of serum was diluted with 3 ml of 0.02M Tris buffer (pH 7.4), 0.5 ml of 0.2% heparin, and 0.5 ml of 0.4M $CaCl_2$, sequentially. After standing overnight under refrigeration, the samples were centrifuged at 4 C, and the clear HDL portion removed quantitatively to another vessel. If the solutions were not clear (serum with very high VLDL + LDL content), additional heparin and calcium were added to obtain a clear supernatant. The precipitates were resuspended in 0.5 ml of water and then diluted to 20 ml with 2:1 chloroform:methanol and shaken. After an overnight extraction, aliquots were dried, re-extracted with isopropanol, and analyzed for cholesterol. To the ca. 4 ml of aqueous HDL solution, we added 7 ml absolute methanol, with mixing, let it stand for about 4 hr, and then added 14 ml of chloroform with mixing. After extracting overnight, most of the clear upper-phase was aspirated and discarded. The remainder was diluted to 20 ml with absolute methanol and then centrifuged. Aliquots of the clear supernatant were dried, extracted with isopropanol, and analyzed for cholesterol. By these methods, we observed 100 ± 10% recoveries. Note! – if either the precipitate or aqueous HDL fractions were dried, they became very refractory to extraction.

RESULTS

Single Dose Studies

Evaluation of U-41,792 emulsion: Single doses of U-41,792 emulsion effectively reduce serum cholesterol levels, heparin precipitating lipoproteins (HPL), and the HPL/cholesterol ratio when given either orally (Table I) or intravenously (Table II). These activities were comparable to those observed in which U-41,792 activity was measured 18 hr after four daily doses administered in aqueous vehicle (1). Activity was equally good with autoclaved and nonautoclaved emulsion. Thus, U-41,792 is stable to emulsion preparation, including autoclaving (121 C for 15 min). Active oral doses increased fresh liver weights (Table I). Unexpectedly, we also noted that the orally administered cottonseed oil emulsion was hypocholesterolemic by itself (emulsion control) as compared to the undosed rats (negative controls) (Table I). The cottonseed oil probably was responsible for this activity (dosage 3000 mg/kg), but we cannot rule out the possible influence of the lecithin (240 mg/kg) or Pluronic® F68 (30 mg/kg) dosages also administered in the emulsion.

Time-activity responses: The effects on

serum cholesterol of one oral or intravenous dose of U-41,792 with time is illustrated in Figure 1. The responses following either route of administration were markedly similar. During the first 10 hr the response to intravenous doses were parallel to and separated by 4 hr from the response to oral doses. An allowance of 4 hr for absorption of drug from the digestive tract is reasonable. Unfortunately, we did not bleed groups after 6 hr, so significant reductions of cholesterol were first observed at 8 hr with intravenous and at 10 hr with oral doses. These results indicate that the U-41,792 molecule is rapidly absorbed and possibly acts as the intact molecule. After 10 hr, the activities of oral and intravenous dosages were quite similar and, surprisingly, persisted for at least 4 days. Control cholesterol levels were depressed, particularly at 48, 56, and 64 hr after dosing. This response, we presume, was caused by the control cottonseed oil emulsion administered both orally and intravenously. Saline perfused liver weights were not significantly altered at any time period.

Site of cholesterol accumulation: We calculate that at least 5 mg of cholesterol disappears from the hypercholesterolemic rat blood within 16 hr after one dose of U-26,719. Among tissues examined, only livers of drug-treated rats showed significant accumulations of cholesterol (Table III). This was accomplished mainly by the average 24% increase in fresh liver weights. These results indicate that the liver is one site of drug action.

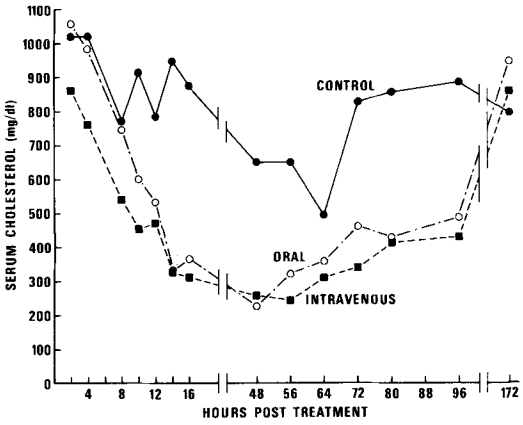

FIG. 1. Effects of a single oral and intravenous 100 mg/kg dose of U-41,792 on serum cholesterol of hypercholesterolemic rats. Each point represents the mean of six rats.

Multiple Dose Studies

Effects on serum: Activities of 6, 25, and

TABLE III

Cholesterol in Serum and Tissues of the Hypercholesterolemic Rat 16 Hours After One U-26,719 Treatment[a]

	Control	U-26,719 50 mg/kg	% S.D.
Serum			
Cholesterol (mg/dl)	402	145[b]	15.8
Total blood cholesterol[c] (mg)	8.8	3.2	
Liver			
Wet weight (g)	2.79	3.47[b]	11.0
Cholesterol (mg/g)	15.4	18.6	12.6
Total cholesterol (mg)	42.4	61.2[b]	12.8
Spleen			
Weight (g)	0.28	0.26	
Cholesterol (mg/g)	4.89	5.09	5.6
Total cholesterol (mg)	1.35	1.35	18.3
Adrenals			
Weight (g)	0.16	0.15	
Cholesterol (mg/g)	45.2	44.7	17.1
Total cholesterol (mg)	0.74	0.66	32.1
Epididymal fat			
Weight (g)	0.21	0.23	
Cholesterol (mg/g)	1.98	1.94	16.1
Total cholesterol (mg)	0.42	0.45	13.2

[a]Five rats (65 ± 6 g) per group.

[b]Significantly different from control, $P \leq .05$.

[c]Calculated. Based upon blood volume of 6.7% of body weight and serum as 50% of the blood.

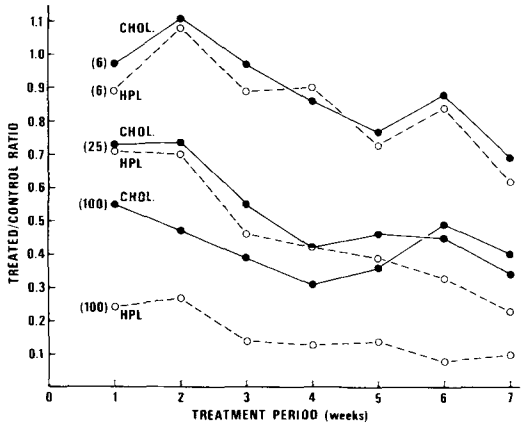

FIG. 2. Effects of multiple doses of U-41,792 on serum cholesterol and heparin precipitating lipoproteins of hypercholesterolemic rats. Numbers in parentheses are daily mg/kg doses. Treated/control ratios of mean responses of seven control rats, eight at 6 mg, ten at 25 mg, and nine at 100 mg/kg were plotted.

100 mg/kg/day U-41,792 doses after each week for 7 wk are shown graphically in Figure 2. All dose levels demonstrated more activity after 7 wk than was shown after 1 wk. Statistically significant reductions of cholesterol and HPL levels were obtained with 6 mg/kg only after 7 wk, but 25 and 100 mg/kg doses were active throughout the 7 wk. HPL levels were reduced significantly more than cholesterol levels throughout the 7 wk with 100 mg U-41,792, only after 5 wk with 25 mg/kg, and at no time with 6 mg/kg doses. Significantly greater reductions of HPL relative to total cholesterol levels indicate shifts of cholesterol to high density lipoproteins which are not precipitated by heparin. After 7 wk, livers of the drug treated rats weighed the same or less than controls. Most strikingly, livers of treated rats were a more normal reddish-brown color and were distinguished easily from the very pale livers of the controls. These results indicate that hypobetalipoproteinemic activities of U-41,792 are enhanced by multiple doses and that these treatments also ameliorate the effects of cholesterol-cholic acid feedings upon the rat liver.

Effects on livers: In hypercholesterolemic rats at 50, 100, and 200 mg/kg/day for 1, 2, 4, and 7 wk, U-41,792 effectively reduced serum cholesterol and HPL levels at all doses (Table IV). Also, all doses, except 50 mg/kg/day for 1 wk (cholesterol and HPL T/C ratios both = .57), significantly reduced HPL levels more than cholesterol levels. Significant decreases in weight gains were noted only with 200 mg/kg/day after 4 and 7 wk. Food intakes were not significantly affected.

TABLE IV

Effects of Multiple Doses of U-41,792 on Serum Cholesterol and Heparin Precipitating Lipoproteins (HPL)

	Control		50		100		200		% S.D.	
	Chol. (mg/dl)	HPL ($A_{680}X10^3$)	Chol. (T/C[a])	HPL (T/C[a])	Chol. (T/C[a])	HPL (T/C[a])	Chol. (T/C[a])	HPL (T/C[a])	Chol.	HPL
1 Week	411	613	.57[b]	.57[b]	.36[b]	.15[b]	.41[b]	.07[b]	17.2	40.8
2 Weeks	435	708	.29[b]	.12[b]	.41[b]	.05[b]	.48[b]	.04[b]	26.3	61.2
4 Weeks	599	466	.61[b]	.11[b]	.43[b]	.08[b]	.61[b]	.11[b]	18.7	44.7
7 Weeks	328	481	.36[b]	.21[b]	.29[b]	.05[b]	.48[b]	.06[b]	19.3	48.3

[a]Treated/control ratios, six rats per group.
[b]Significantly different from controls, $P \leq .05$.

TABLE V

Effects of Multiple Doses of U-41,792 on Dry Liver Weight (Expressed as % of Body Weight)[a]

	Control	50	100	200	% S.D.
1 Week	1.76	1.72	1.97[b]↑	2.18[b]↑	8.9
2 Weeks	2.04	2.18	2.57[b]↑	2.84[b]↑	8.9
4 Weeks	2.65	2.39	2.27	2.65	13.0
7 Weeks	2.95	2.66[b]↓	2.27[b]↓	2.66[b]↓	8.4

[a]Six rats per group.

[b]Significantly different from controls, $P \leq .05$.

TABLE VI

Effects of Multiple Doses of U-41,792 on Liver Cholesterol (mg/g dry liver)[a]

	Control	U-41,792 50 mg/kg	U-41,792 100 mg/kg	U-41,792 200 mg/kg	% S.D.
1 Week	175	142[b]	123[b]	103[b]	8.9
2 Weeks	202	148[b]	114[b]	76[b]	8.3
4 Weeks	251	195	136[b]	81[b]	22.0
7 Weeks	306	170[b]	126[b]	74[b]	14.6

[a]Six rats per group.

[b]Significantly different from control, $P \leq .05$.

TABLE VII

Effects of Multiple Doses of U-41,792 on Liver Total Lipids (mg/g dry liver)[a]

	Control	U-41,792 50 mg/kg	U-41,792 100 mg/kg	U-41,792 200 mg/kg	% S.D.
1 Week	501	449	455	479	10.0
2 Weeks	550	522	547	561	8.0
4 Weeks	605	600	557	556	7.6
7 Weeks	681	624[b]	585[b]	592[b]	4.9

[a]Six rats per group.

[b]Significantly different from control, $P \leq .05$.

Liver weights (Table V) were significantly increased after 1 and 2 wk, unchanged after 4 wk, and reduced after 7 wk of treatment with 100 and 200 mg/kg U-41,792. Hepatomegaly was not observed with 50 mg/kg/day at any time period, and the livers weighed less than controls after 7 wk. The livers of all treated animals were distinguished easily from controls by their more normal color.

Liver lipid analyses revealed that U-41,792 treatments decrease liver cholesterol concentrations at all time periods (Table VI), but either have no effects upon total lipid concentration or reduce lipid concentrations 10-15% from controls after 7 wk (Table VII). Since liver cholesterol was reduced whereas liver lipids either remained unchanged or increased (rats with hepatomegaly), either triglycerides and/or phospholipids must have been increased by U-41,792 treatment.

Effects on lipoproteins: Cholesterol analyses of the heparin precipitates and supernates of sera following one week treatment illustrated the activity of U-41,792 on serum lipoproteins (Fig. 3). As dosage increased, levels of high density α-lipoproteins increased and lower density β- and preβ-lipoproteins decreased. The most striking effects were observed in the sera of the rats given 200 mg/kg/day. Levels of β- and preβ-lipoproteins were reduced to those observed in normal rats, whereas α-lipoproteins were increased to levels 2½ times those of normal rats.

DISCUSSION

A new type of hypolipidemic activity which

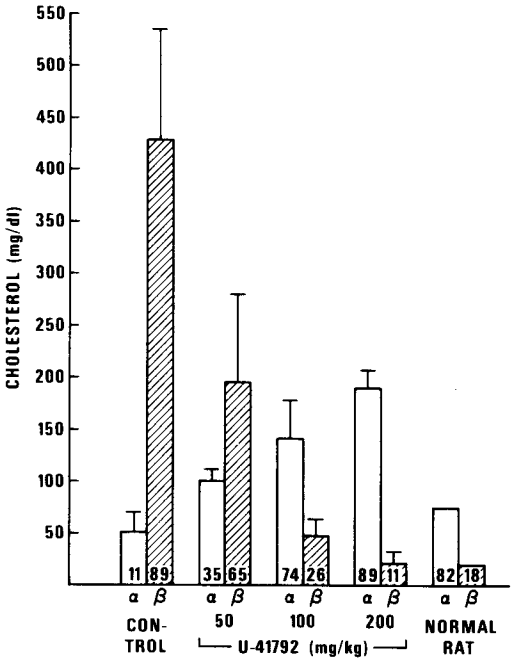

FIG. 3. α- and β- + preβ-lipoprotein cholesterol (± Standard Deviation) after seven daily doses of U-41,792. Numbers in bars = % of total cholesterol. Six hypercholesterolemic rats were in each group. Normal rat serum was a pooled sample.

we have designated hypobetalipoproteinemic has been demonstrated. In cholesterol-cholic acid fed rats, this activity results in a dramatic reduction of the lower density lipoproteins that are precipitated by heparin (HPL) with a concomitant increase in high density lipoproteins not precipitated by heparin. Operationally this activity is manifested by a reduction in HPL/cholesterol ratios and was observed following single or multiple doses of U-41,792.

The pattern of responses following single oral or intravenous doses indicated that the U-41,792 molecule is rapidly absorbed from the intestinal tract. Dramatic reductions of serum cholesterol were observed within 10 hr following a single dose which persisted for at least 4 days. Therefore, it was not surprising to determine that activity was enhanced by multiple doses. The liver is at least one site of action of U-41,792, since we noted decreased liver cholesterol and lipid accumulation following a chronic 7 wk administration of U-41,792. Furthermore, hepatomegaly was observed following treatment for 1 or 2 wk and after single dosages. From these and other observations, we speculate that by some unknown mechanism the U-41,792 molecule causes a rapid accumulation of loosely bound, cholesterol-carrying, lower density lipoproteins in the liver. Concomitantly, high density lipoprotein concentrations increase in the sera. Ultimately, continued treatments cause an elimination of excess cholesterol and lipid from the liver.

It is impossible to extrapolate activity in our animal models to activity in humans. In our hyperlipemic rat model, the lower density lipoproteins are predominantly cholesteryl ester-rich VLDL (9), very different from those that occur in human hyperlipoproteinemia (with the possible exception of type III hyperlipoproteinemia). The compositions of high densiy lipoproteins in our models are also unlike those of any human lipoprotein (2). Nevertheless, the unique activities of this hypobetalipoproteinemic agent in animal models may provide a useful tool for exploring the complex and relatively unknown area of lipoprotein catabolism.

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An Overview of the Biochemical Pharmacology of Probucol

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ABSTRACT

Probucol was effective in lowering serum total cholesterol in mice at dietary levels as low as 0.0075%. It was also effective after a single 100 mg/kg I.V. dose in mice. The incorporation of acetate-^{14}C into liver lipids of rats and mice was not significantly affected by probucol, although the results, especially in mice, make it impossible to rule out such an effect. Cholesterol absorption was estimated in rats using a dual isotope technique. The observed reductions were not statistically significant. Several liver enzyme activities were determined after probucol treatment in rats, and a significant elevation (32%) was observed in only one, glutamic dehydrogenase. Serum cholesterol was lowered markedly in cholesterol-fed cynomolgus monkeys by probucol. There was no effect on the excretion of neutral steroids and the observed increase in fecal bile acids after drug treatment could not be confirmed statistically.

INTRODUCTION

Probucol was shown to have hypocholesterolemic properties in mice and rats and was selected for further investigation (1). The compound was also active in monkeys, and in studies in rodents no evidence for any effect on the biosynthesis of cholesterol was found (2). Clinical studies have confirmed activity in humans (3,4). The present study explores further aspects of the nature of the lipid-lowering effect of probucol in animals.

MATERIALS AND METHODS

Male rats (Wistar strain, Harlan Industries, Cumberland, IN, for enzyme study; Sprague-Dawley strain, Spartan Farms, Haslett, MI, for radiochemical studies) and mice (Swiss-Webster strain, Laboratory Supply Co., Inc., Indianapolis, IN) were used. Experimental drugs were added to commercial rodent meal (Ralston-Purina Co., St. Louis, MO) unless otherwise indicated. For I.V. administration, probucol was dissolved in Neobee M-5 (PVO International, Inc., Boonton, NJ) and incorporated in an emulsion (5). The experimental monkey diet was prepared by mixing 5 g of cholesterol, 198 g of lard and 200 ml of hot water with 1 kg of ground commercial monkey chow (Ralston-Purina Co., St. Louis, MO). Male monkeys (*Cynomolgus fasicularis*) were given the diet at a rate of 4% of body wt per day. Probucol was administered as an aqueous suspension by nasogastric intubation at a dose of 150 mg/kg b.i.d.

The radiochemicals were obtained from Amersham/Searle Corp., Des Plaines, IL. The radiochemical analyses were performed essentially as described previously (2).

Serum (6) and liver (7) cholesterol, liver glycogen (8), catalase (9), glutamic dehydrogenase (10), glucose-6-phosphatase (11), lactic dehydrogenase (12), and glutamic-pyruvic transaminase (13) were determined using reported methods. Fecal neutral steroids (14) and bile acids (15) were determined gas chromatographically. Cholesterol absorption was estimated using a dual isotope technique (16).

RESULTS

The cholesterol-lowering activity of probucol was readily demonstrated in mice (Table I). Reductions in serum cholesterol ranged from 22-74%. The smallest reduction of 22% was associated with the lowest level of drug in the diet, 0.0075%. The actual dose of drug is dependent on food consumption, and in the present experiment, the 0.03% level corresponded to about 50-60 mg/kg/day, with the other groups being proportionate.

TABLE I

The Effect of Different Dietary Levels of Probucol on Serum Cholesterol in Mice[a]

Percent drug in diet	Percent reduction of serum cholesterol
0.125	73
0.06	74
0.03	40
0.015	31
0.075	22

[a]Groups of 12 male mice, 20-25 g, were treated for 4 wk. All reductions were statistically significant; $p<0.01$.

TABLE II

Effect of Probucol Administered Intravenously in Mice[a]

Hours after injection	Serum cholesterol[b] (mg/dl)		Liver cholesterol[b] (mg/g)	
	Control	Probucol	Control	Probucol
3	121 ± 17	100 ± 16	2.55 ± 0.23	2.75 ± 0.10
6	121 ± 18	89 ± 19[c]	2.34 ± 0.32	2.68 ± 0.13[c]
16	130 ± 20	84 ± 12[d]	2.64 ± 0.17	2.84 ± 0.16
30	137 ± 26	56 ± 24[d]	2.98 ± 0.13	3.33 ± 0.43

[a]Groups of four male mice, 30-35 g, were injected I.V. with 0.1 ml/20 g body wt of an emulsion containing 20 mg/ml probucol.

[b]Mean ± SD.

[c]Significantly different from control value; $p<0.05$.

[d]Significantly different from control value; $p<0.01$.

TABLE III

The Determination of Cholesterol Absorption in Rats Using a Dual Isotope Method[a]

	Serum cholesterol[b] (mg/dl)	Percent absorption[b,c]
Control	78 ± 6	63.1 ± 14.8
Probucol	58 ± 7[d]	50.6 ± 14.0
Clofibrate	56 ± 6[d]	53.9 ± 11.2

[a]Groups of 12 male rats, 210-220 g, were given 0.25% dietary drug until sacrifice (12 days). Sixty-eight hours before sacrifice 3.5 μCi each of 4-^{14}C-cholesterol (oral) and 1,2-^{3}H-cholesterol (I.V.) was administered.

[b]Mean ± SD

[c]$\text{Percent absorption} = \dfrac{\%\text{ total } ^{14}\text{C in serum} \times 100}{\%\text{ total } ^{3}\text{H in serum}}$

[d]Significantly different from control value; $p<0.01$.

Probucol was also active when administered I.V. (Table II). A single 100 mg/kg dose was sufficient to elicit a significant reduction in serum cholesterol in 6 hr. The maximum observed reduction of 59% occurred at 30 hr.

A technique involving the simultaneous administration of 1,2-^{3}H-cholesterol I.V. and 4-^{14}C-cholesterol orally was used to estimate the effect of probucol and clofibrate on cholesterol absorption in rats (Table III). The administration of both drugs at the 0.25% level in the diet for twelve days resulted in similar and significant reductions in serum cholesterol of ca. 25%. The observed reduction in cholesterol absorption of ca. 20% was not statistically significant.

The incorporation of acetate-^{14}C into liver lipids was determined in both mice (Table IV) and rats (Table V). Probucol did not induce a significant inhibition in either species, although the observed difference approached 50% in mice. Clofibrate was tested also in rats and induced a highly significant reduction in the specific activity of the liver sterol fraction.

The effect of probucol and clofibrate on the activity of several liver enzymes and on liver glycogen was determined after a 2-wk treatment period (Table VI). Probucol induced a statistically significant elevation of glutamic dehydrogenase but had no significant effect on the other enzymes studied or on liver glycogen levels. Clofibrate treatment resulted in higher activities for four of the five enzymes studied and reduced liver glycogen levels.

The consumption of a diet containing 0.36% cholesterol and 14% lard by cynomolgus monkeys resulted in serum total cholesterol levels of nearly 1000 mg/dl after 10 wk. Treatment with probucol (150 mg/kg b.i.d.) resulted in ca. a

TABLE IV

Effect of Probucol on Incorporation of Acetate-1-^{14}C into Liver Lipids of Mice

	Activity (x 10^3)[b]	
	Control	Probucol
Liver nonsaponifiable material (dpm/g tissue)	3.08 ± 1.36	1.55 ± 1.10
Liver sterols (dpm/g tissue)	1.52 ± 0.85	0.78 ± 0.64
Liver sterols (dpm/mg cholesterol)	0.57 ± 0.33	0.30 ± 0.23

[a]Male 25-30 g mice were given 0.06% dietary probucol for 3 days. 4.0 μCi (2.0 μmol) of sodium acetate-1-^{14}C were injected I.V. After 20 min, the animals were sacrificed.

[b]Mean ± SD.

TABLE V

Effect of Clofibrate and Probucol on the Incorporation of 1,2-^{14}C-Acetate into Liver Sterols[a]

Groups	Serum cholesterol (mg/dl)	Liver cholesterol (mg/g)	Specific activity (dpm/mg sterol x 10^3)
Control	64.1 ± 6.1	2.27 ± 0.16	2.46 ± 0.64
Clofibrate	46.9 ± 5.6[b]	1.83 ± 0.09[b]	0.87 ± 0.04[b]
Probucol	35.9 ± 4.1[b]	2.29 ± 0.17	2.23 ± 1.01

[a]Groups of 8 male rats, 170-180 g, received 0.125% dietary drug for two weeks. Sodium acetate-1,2-^{14}C (10μCi/100 g body weight) was injected I.V., and the animals were sacrificed 4 hr later. Data are expressed as mean ± SD.

[b]Significantly different from control value; $p<0.05$.

TABLE VI

Effect of Probucol and Clofibrate on Rat Liver Enzyme Activities and Glycogen Levels[a]

catalase (μmoles H_2O_2/mg liver/min)	63.8 ± 5.8	68.2 ± 8.1	76.0 ± 5.8[c]
lactic dehydrogenase (units/mg liver)[b]	320 ± 32	327 ± 34	504 ± 38[c]
glutamic dehydrogenase (units/mg liver)[b]	2.17 ± 0.45	2.87 ± 0.47[c]	3.52 ± 0.56[c]
glucose-6-phosphatase (mmoles PO_4/g liver/hr)	0.40 ± 0.02	0.40 ± 0.02	0.42 ± 0.06
glutamate-pyruvate transaminase (mmoles pyruvate/g liver/hr)	1.64 ± 0.72	2.06 ± 0.42	2.61 ± 0.73[c]
glycogen (mg/g liver)	49.9 ± 9.5	46.2 ± 9.2	35.7 ± 7.3[c]

[a]Each value is an average of six determinations (five in probucol group) ± SD. Male 90-120 g rats were maintained on 0.25% dietary drug for two weeks.

[b]Unit = Δ absorbance at 340 nm of 0.001 in 1 min.

[c]Statistically different from control value, $p<0.05$.

50% decrease in 2 wk (Fig. 1). Fecal steroids were determined over a period ranging from 3 wk before to 5 wk after the initiation of treatment. There was no discernible shift in the excretion of neutral steroids as the result of probucol treatment (Fig. 2). The high value just before day 0 was due to a single 0.86 g dose of β-sitosterol 6 days before drug treatment. Recovery was estimated to be 76%. No corrections were applied to the data. There was an apparent increase in the excretion of fecal bile acids (Fig. 2), some quantities being double the pretreatment values after 2-4 wk of treatment. However, the high variability makes it impossible to draw a definitive conclusion.

DISCUSSION

Probucol has previously been shown to lower serum cholesterol in various species, with little or no effect on liver size or lipid levels (2). In the present work, a dose-response relationship was observed in mice. The maximal effect was achieved with the 0.06% dietary level.

The drug was also effective I.V., and in a rather short time. Biliary excretion of drug following this mode of administration could have an effect in the intestinal tract but considering the short period of time involved, it seems unlikely that effects such as decreased absorption or increased excretion could be of prime importance in this study. In a study in rats involving oral drug administration, no significant effect on cholesterol absorption was observed.

No effect was observed in earlier tests using radioactive precursor incorporated into liver lipids as an estimate of biosynthetic inhibition (2). Further experiments reported here tend to substantiate those results, particularly in rats, where experimental data are routinely within a few percent of control values. Conversely, in mice, incorporation of acetate-^{14}C, but not

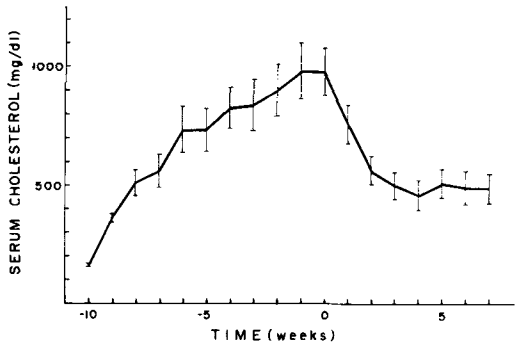

FIG. 1. Effect of probucol on serum total cholesterol in experimentally induced hypercholesterolemic cynomolgus monkeys. Oral treatment with 300 mg/kg/day probucol started at time zero. Vertical lines represent S.E.

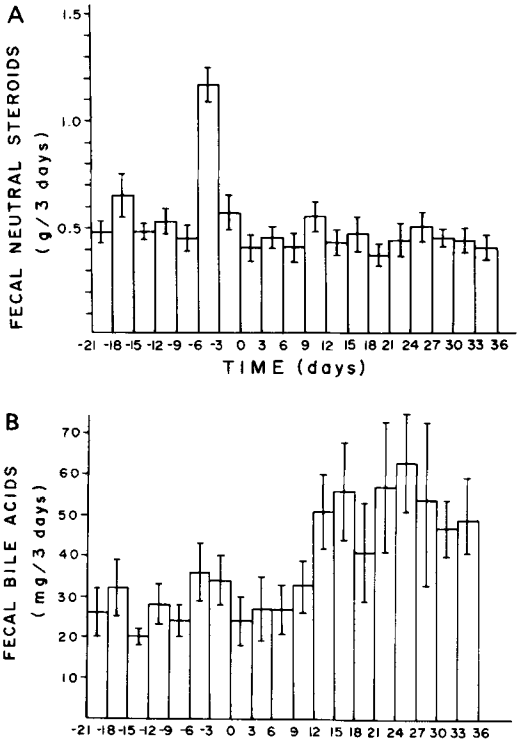

FIG. 2. Effect of probucol on fecal neutral steroids (A) and bile acids (B) in experimentally induced hypercholesterolemic cynomolgus monkeys. Oral treatment with 300 mg/kg/day probucol started at time zero. Vertical lines represent S.E.

mevalonate, into liver sterols has often been substantially lower in probucol-treated animals. Unfortunately, this finding could not be verified statistically because of the high variability.

Elevated liver glutamic dehydrogenase levels were observed after probucol treatment, whereas several other enzyme activities were not significantly affected. Increased catalase activity has been associated with clofibrate treatment (17). The clofibrate-induced increase in GDH and GPT activities (Table VI) is not in agreement with the data of Platt and coworkers (18,19); however, decreased liver glycogen and increased lactic dehydrogenase activity were reported by that group.

The degree of hypercholesterolemia achieved in cynomolgus monkeys was similar to that reported by others (20). It is not known whether any feedback inhibition exists under these conditions. An effect on biosynthesis cannot be ruled out, but the marked reduction of these extremely high serum cholesterol levels is suggestive of an effect on formed cholesterol. Probucol was active in cholesterol-fed rabbits, but only at a relatively high dose, 1% in the diet (21). The observed increase in fecal bile acids in the present study, though not statistically significant, may be at least partly responsible for the action of the compound. An increase in fecal bile acids, as well as a reduction of cholesterol synthesis and absorption, was observed in humans (22).

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Studies on the Mechanism of Action of Halofenate[1]

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ABSTRACT

This paper reviews most of the preclinical studies on the mode of action of halofenate, an established hypolipidemic-hypouricemic agent in man. In yeast cultures and in isolated rat adipocytes, halofenate was found to inhibit the conversion of pyruvate to acetyl CoA. While pyruvate dehydrogenase was inhibited in vitro, halofenate also inhibited the activity of various other isolated enzymes. In rats maintained on halofenate in the diet (0.02-0.10%) for 2-14 days, there were 20-40% decreases in plasma cholesterol, triglycerides, phospholipids, and free fatty acids. Inhibition of liver HMG-CoA reductase does not appear to account for the hypocholesterolemic effect, and activation of mitochondrial α-glycerophosphate dehydrogenase does not explain the hypotriglyceridemic action. Kinetic measurements of the serum appearance and disappearance of triglycerides in drug-treated rats suggest that the hypotriglyceridemic activity is due to a net inhibition of hepatic triglyceride synthesis. Reduction of very low density lipoprotein (VLDL) and high density lipoprotein (HDL) levels in rats with sucrose-induced hyperlipidemia and normalization of the altered apolipoprotein profiles are in accord with the effects of halofenate on plasma triglyceride and cholesterol levels. The reduced insulin-to-glucagon ratio observed in Zucker obese hyperlipemic rats is also consistent with halofenate's hypotriglyceridemic activity. Preliminary experiments in rats on the mechanism of its hypoglycemic activity, observed in some diabetic hyperlipidemic patients, indicate that halofenate acts differently than conventional oral hypoglycemic agents. Some, but not all, of the effects of halofenate were observed with clofibrate at two to ten times higher levels.

[1]The list of literature references on halofenate cited in this paper is not a complete one. Since 1970, there have been over 100 publications on halofenate of which 20 relate to mechanism of action studies in laboratory animals or man. Fifty are primarily concerned with clinical hypolipemic and hypouricemic activity and the balance on other aspects of its properties.

INTRODUCTION

The question of whether plasma lipid lowering agents exert their action by affecting a single enzyme or by influencing several enzymes or biochemical processes has been investigated for many years in a number of laboratories. In the case of clofibrate, for example, there are a variety of mechanisms which could account for its hypotriglyceridemic and hypocholesterolemic activity (1-6). How these different mechanisms relate to one another and to the clinical effects of clofibrate is not yet established.

The intent of this paper is to outline the highlights of several preclinical investigations on the mode of action of the hypolipidemic agent halofenate [2-acetamidoethyl(p-chlorophenyl) (m-trifluoromethylphenoxy) acetate]. Some of these studies were carried out by members of the Department of Biochemistry at the Merck Sharp & Dohme Research Laboratories while others were undertaken by collaborating investigators. Data sources include unpublished studies and personal communications as well as published reports on halofenate.

Figure 1 shows the structure and nomenclature for halofenate (MK-185). Like clofibrate, halofenate produces reductions primarily in serum triglycerides but less frequently in serum cholesterol. Like probenecid, halofenate produces consistent reductions in uric acid. The changes in metabolic parameters at the end of 48 wk of treatment with 1 g of halofenate in a single daily oral dose are shown in Table I. These data indicate the order of the hypolipemic activity of halofenate and are representative of the results obtained in various trials during 5 yr of clinical evaluation. A few selected papers on the clinical effects of halofenate are given in the reference section (7-13).

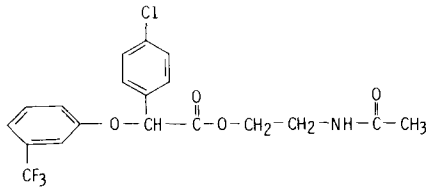
RESULTS AND DISCUSSION

Isolated Systems

In the initial mechanism studies, the effect of halofenate on lipid synthesis in cultures of *Saccharomyces cerevisiae* was investigated. Since the enzymatic steps of lipid biosynthesis are similar in yeast and mammalian cells, it was reasoned that the simpler system might identify a primary site of action more readily (14). The results of this line of investigation are summarized in Table II. The free acid of halofenate

(HFA), the active moiety (15), was found to inhibit yeast growth reversibly; at 0.34 mM, 50% inhibition of growth was achieved. The inhibitory effect of HFA was prevented by adding 0.35 mM oleic or 3.7 mM acetic acids to the culture medium. Pyruvic acid at much higher concentrations (to 72 mM) could not by-pass the block. HFA inhibited the conversion of ^{14}C-glucose and ^{14}C-pyruvate to labelled fatty acids and sterols, but conversion of ^{14}C-acetate to lipids was not affected. These findings taken with the inhibition of the decarboxylation of pyruvate-1-^{14}C to $^{14}CO_2$ strongly suggest that in the yeast system, growth and lipogenesis are inhibited by a block at the pyruvate dehydrogenase complex.

In extending these observations to animal tissues, experiments were carried out with isolated adipocytes prepared from rat fat pads (16). While HFA inhibited the uptake of ^{14}C-glucose and ^{14}C-pyruvate to the same extent as it blocked their incorporation into fatty acids, it did not inhibit the uptake of leucine, palmitate, or 2-deoxyglucose, suggesting that it was not acting as a nonspecific transport inhibitor. That the drug inhibited lipogenesis was demonstrated in experiments wherein HFA inhibited conversion of pyruvate-2-^{14}C to labelled fatty acids to the same extent as it blocked the decarboxylation of pyruvate-1-^{14}C to $^{14}CO_2$ (Table III). A comparison of the activity of HFA with three established



Halofenate (MK-185)

2-Acetamidoethyl (p-chlorophenyl) (m-trifluoromethylphenoxy) acetate

FIG. 1. Structure and nomenclature of halofenate.

lipogenesis inhibitors—hydroxycitrate, kynurenate and cerulenin—shows that at levels which inhibit fatty acid synthesis 50%, inhibition of pyruvate decarboxylation decreased with the proximity of cytoplasmic enzyme to pyruvate dehydrogenase (16). These findings further suggest that inhibition of enzymes involved in fatty acid biosynthesis in the cytoplasm results in product inhibition of mitochondrial pyruvate dehydrogenase. While the exact site of action of halofenate on lipogenesis in adipocytes cannot be determined unequivocally from these studies, the pattern of data suggests that HFA acts prior to the conversion of citrate to acetyl CoA, narrowing the site to pyruvate dehydrogenase or citrate synthetase.

In contrast to the results obtained in yeast and adipocytes, halofenate-induced inhibition of lipid synthesis in isolated liver systems was

TABLE I

Changes in Metabolic Parameters at the End of 48 Weeks of Halofenate Treatment

Measurement[a]	Pretreatment mean	Change from pretreatment mean	% Change
Body weight (lbs)	177.4	+1.2	+0.7
Fasting blood sugar (mg %)	116.4	-8.0	-6.9
Serum triglycerides (mg %)	399.4	-93.4	-23.4
Serum cholesterol (mg %)	293.3	-15.3	-5.2
Serum uric acid (mg %)	6.87	-2.32	-33.8

[a]328 patients, primarily type IV.

TABLE II

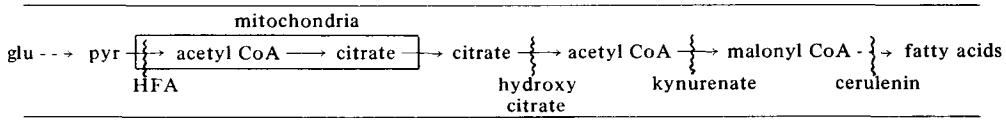
Effect of Halofenate Free Acid (HFA) on Growth and Lipid Synthesis in *S. cerevisiae*[a]

1. HFA inhibits yeast growth 50% at 0.34 mM.
2. Inhibitory effect prevented by added oleate or acetate but not pyruvate.
3. HFA inhibits conversion of glucose-U-^{14}C or pyruvate-2-^{14}C, but not acetate-2-^{14}C, into labelled fatty acids and sterols.
4. HFA inhibits conversion of pyruvate-1-^{14}C to $^{14}CO_2$.
5. In vitro yeast PDH is inhibited 50% by 0.4 mM HFA.

[a]Adapted from the report of Greenspan and Germershausen (14).

TABLE III

Effect of HFA on Lipid Synthesis in Isolated Rat Adipocytes[a]



	Compound	Site	% Inhibition pyr-2-^{14}C to fatty acids	% Inhibition pyr-1-^{14}C to $^{14}CO_2$
1.5 mM	HFA	[PDH]	50	50
10.0 mM	Hydroxy citrate	ATP-citrate lyase	50	40
10.0 mM	Kynurenate	Acetyl CoA carboxylase	50	27
0.12 mM	Cerulenin	Fatty acid synthetase	50	16

[a]Adapted from the report of Greenspan et al. (16).

TABLE IV

Effect of Halofenate (free acid) on the Activity of Various Enzymes in vitro[a]

Enzyme	Source	Conc. (mM) of HFA producing 50% inhibition
Pyruvate dehydrogenase	Yeast	0.4-3.0
	Pig heart	2.0
Acetyl CoA carboxylase	Chicken liver	1.5
	Yeast	0.2
Citrate cleavage enzyme	Rat liver	2.0
HMG CoA reductase	Yeast	0.1
	Rat liver	>1.0
Fatty acid synthetase	Rat liver	0.3
α-Glycerophosphate dehydrogenase	Rat liver	0.3
Glucose 6-phosphate dehydrogenase	Yeast	5.0
Lactic acid dehydrogenase	Rabbit muscle	9.2
Prostaglandin synthetase	Sheep seminal vescicle	0.2
Insulinase	Rat liver	1.0

[a]Taken from Vol. I of the preclinical evaluation of halofenate, Merck Sharp & Dohme Research Laboratories, March 22, 1974.

associated with an inhibition of protein synthesis. Thus, in liver slices from normal adult rats, Morris 7777 hepatoma and fetal rat liver, or in primary rat fetal hepatocytes grown in culture, neither halofenate nor clofibrate inhibited lipid synthesis out of proportion to the inhibition of total protein synthesis (Greenspan; Weinstein and Steinberg, unpublished results). With isolated hepatocytes from adult rats, inhibition of lipogenesis was also associated with inhibition of protein synthesis (17; Gould and Shrewsbury, unpublished results). In hepatocytes, halofenate was also shown to stimulate fatty acid metabolism, suggesting the drug causes their displacement from albumin (17). While liver mitochondrial preparations from rats maintained on halofenate or clofibrate showed a stimulation of cholesterol 26-^{14}C oxidation to $^{14}CO_2$, an effect apparently related to an increase in liver size common to several phenoxyacetic acid structures (18-19), the significance of this activity in terms of understanding its hypolipemic activity is not clearly established (1).

Another approach undertaken to determine the mode of action of plasma hypolipemic drugs has been to evaluate the compounds in vitro as inhibitors of isolated enzymes involved in the biosynthesis of triglycerides and sterols. As in the case of clofibrate, this does not seem to represent a fruitful line of endeavor for halofenate. Table IV shows that HFA inhibits the activity of enzymes involved in a variety of biochemical reactions. Moreover, enzymes not

TABLE V

Effect of Halofenate on Plasma Lipid Parameters in Rats[a]

% of Halofenate in diet (9 days)	Body weight gain (gm)	% Decrease from control[b]			
		Chol.	TG	PL	FFA
0 (Control)	48	-	-	-	-
0.00625	46	12	12	10	0
0.0125	50	24	18	18	23
0.025	50	31	29	24	38
0.05	49	38	43	31	34

[a]Adapted from the report of Gilfillan et al. (24).

[b]Chol. = cholesterol, TG = triglycerides, PL = phospholipid, FFA = free fatty acids.

TABLE VI

Effect of Dietary (0.05%) Halofenate on Rat Liver HMG-CoA Reductase Activity and Sterol Synthesis in Vitro

Exp. no.	Animal group	Percent change in			
		Serum cholesterol	HMG-CoA reductase	Incorporation of ^{14}C into digitonide precipitable sterols by liver homogenates from	
				^{14}C-acetate	^{14}C-mevalonate
1	midnight rats - 7 days	-50	-13	-8	+52
2	midnight rats - 7 days	-26	+2		
3	midnight rats - 2 days	-36	+30		
4	basal rats - 2 days	-27	-26		

directly involved in lipogenesis are also inhibited by millimolar levels of HFA. Clofibrate free acid (CPIB) produced similar results at four to ten times higher levels (data not shown) and the published literature indicates that CPIB inhibits many enzymes and biochemical processes in vitro (3-6,20-23).

Studies in Rats

The in vivo hypolipemic activity of halofenate was first demonstrated in rats given the drug orally (24). As shown in Table V, halofenate treatment (0.05% in the diet for 9 days) produced ca. 35-40% reduction in cholesterol, triglycerides, phospholipids, and free fatty acids in male Holtzman rats (165 g) maintained on a 72% sucrose diet. A small (12%) but statistically significant reduction in cholesterol and triglycerides was found with halofenate at 0.00625% in the diet. In this animal assay, halofenate was about six times more potent than clofibrate as a hypocholesterolemic agent and nearly twice as potent as a plasma hypotriglyceridemic agent.

To determine whether halofenate inhibited pyruvate metabolism, rats maintained on 0.05% dietary halofenate for 10 days were given pyruvate-2-^{14}C by i.p. injection. One hour later they were sacrificed and the livers removed for the isolation of glycogen. The labelled glycogen was hydrolyzed to glucose, which was degraded (25). There was no difference in the distribution of radioactivity in the carbon atoms from ^{14}C-glucose derived from the glycogen of control rats compared to that of the drug-treated animals (Landau, unpublished results). Had conversion of pyruvate-2-^{14}C to labelled acetyl CoA been inhibited, then the percent of ^{14}C in carbon atoms 3 and 4 would have been expected to be lower in the rats given halofenate. While this result, taken with the failure of 4-10% dietary triacetin or sodium acetate to reverse the hypolipemic effect of halofenate, does not confirm that pyruvate dehydrogenase is the site of action for the drug, additional in vivo experimentation is required to validate or invalidate the pyruvate dehydrogenase mechanism.

In view of the important role of HMG-CoA reductase in controlling cholesterol biosynthesis (26), the effect of halofenate on its activity was investigated. Male Sprague Dawley rats, 200-250 g, were given the drug at 0.05 and 0.15% in the diet for 2 and 7 days; enzyme activity was measured in liver extracts at the time of its peak activity (midnight) and at noon, the time of its basal activity (27). Incorporation of 2-^{14}C acetate and 2-^{14}C-mevalonate into sterols was also measured in one of the experiments (28). The data in Table VI

TABLE VII

Comparison of the Effect of Halofenate and Clofibrate on Rat Liver α-Glycerophosphate Dehydrogenase

Drug	% Decrease in plasma triglycerides	Enzyme activity[a]
None	-	53
0.05% Halofenate	42	77 (45% ↑)
0.1% Halofenate	51	92 (74% ↑)
0.1% Clofibrate	23	156 (195% ↑)
0.2% Clofibrate	28	229 (332% ↑)

[a]$\mu l/O_2$/50 mg tissue/hr.

show that 0.05% halofenate lowers plasma cholesterol but does not markedly reduce HMG-CoA reductase activity or the incorporation of labelled acetate or mevalonate into digitonide precipitable sterols (Erickson and Gould, unpublished results). At a level of 0.15% halofenate in 2-day experiments, there was a 40% decrease in food intake and the drop in plasma cholesterol was accompanied by a reduction in HMG-CoA reductase activity and incorporation of both 1-^{14}C-acetate and 2-^{14}C mevalonate into sterols. In LM-cell culture, neither halofenate nor clofibrate (at 12.5 μg/ml) inhibited desmosterol synthesis in assays which detected the HMG-CoA reductase inhibitory activity of 25-hydroxy or 7-keto cholesterol (29; 30 and Mandel, unpublished results).

These results suggest that inhibition of mevalonate formation does not account for the hypocholesterolemic activity of halofenate in the rat, in contrast to results on clofibrate in which a marked and specific inhibition in cholesterol synthesis from 1-^{14}C acetate but not from 2-^{14}C mevalonate was reported (31,32). However, preliminary data from another study led to the opposite conclusion. White had reported that clofibrate regulates hepatic cholesterol biosynthesis by inhibiting the microsomal reduction of HMG-CoA to mevalonate (33). When halofenate was given to 170 g male Sprague-Dawley rats at 0.05% in the diet for 7 days, the reduction of ^{14}C-HMG-CoA to labelled mevalonate was inhibited 52% in assays employing a liver microsomal system (White, unpublished results). Twenty-nine mμmoles of ^{14}C-HMG-CoA was incorporated into mevalonate with preparations from control animals compared to 14.4 mμmoles for the drug-treated rats. Clearly, more experimentation is required to understand the involvement of this key enzyme in the hypocholesterolemic action of halofenate.

With regard to the hypotriglyceridemic effect of halofenate, activation of mitochondrial α-glycerophosphate dehydrogenase was considered because this should decrease the amount of α-glycerophosphate available for triglyceride synthesis. One effect of clofibrate is to induce the synthesis of liver mitochondrial proteins such as the dehydrogenase, and the hypolipemic activity of this drug has been attributed to this (34-36).

Rats (Holtzman, 150 g) were maintained on dietary halofenate or clofibrate at least 7 days prior to enzyme assay. Liver mitochondrial enzymes were assayed by the method of Ruegamer et al. (34). As shown in Table VII under conditions where halofenate was more than four times as potent as clofibrate in lowering triglycerides, clofibrate was about seven times more effective than halofenate in stimulating the mitochondrial α-glycerophosphate dehydrogenase (Huff, unpublished results). It would appear that if the mechanism of the hypotriglyceridemic action of these two drugs is similar, then their effect is probably not mediated by activation of the dehydrogenase. Alternatively, if clofibrate does influence plasma triglycerides because it activates this enzyme, then the mode of action of halofenate should be different.

In another study designed to establish the mode of the hypotriglyceridemic action of halofenate, male Wistar rats (200 g) were fed a fat-free diet containing no drug, 0.02% or 0.10% halofenate for 14 days. They were then given equivalent amounts of either 2-^{3}H- or 1(3)-^{3}H-glycerol i.v., and blood samples were collected from 10 to 150 min later for the analysis of labelled triglycerides (37). Kinetic analysis of the serum appearance and elimination curves of ^{3}H-triglyceride permitted an estimation of total serum ^{3}H-triglyceride formation and fractional turnover rates. The experimental design was similar to that described by Nikkila and Kekki for measuring plasma triglyceride kinetics in man (38). It was found that the total amount of ^{3}H-triglyceride formed

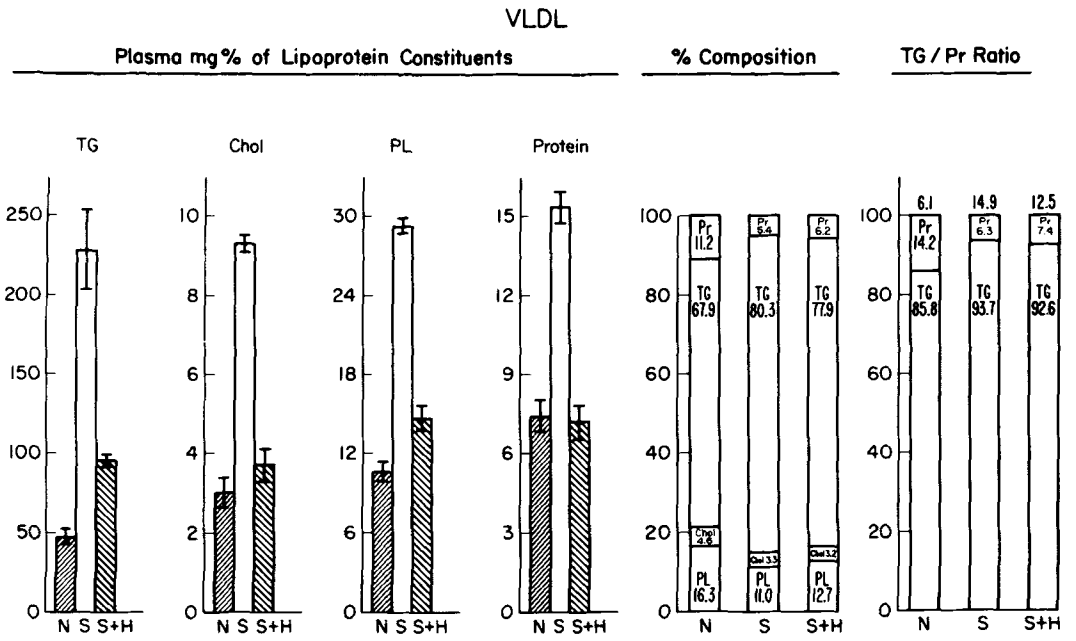


FIG. 2. The abbreviations used are: VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein; TG = triglyceride; Chol = cholesterol; PL = phospholipid, Pr = protein; N = normal diet (for rats maintained on a normal chow diet); S = sucrose fortified semi-purified diet (for rats maintained on the 60% sucrose diet); S+H = sucrose plus halofenate diet (for rats given 0.05% halofenate in the 60% sucrose diet).

from the 2-^{3}H- or 1(3)-^{3}H-glycerol in control rats was similar. Over 95% of the ^{3}H serum triglyceride formed from either substrate circulated in a rapidly turning-over tirglyceride pool ($t_{1/2}$ = 8 min). Treatment of the rats with 0.10% halofenate decreased by about 75% the labelling of serum triglycerides without increasing the fractional turnover rates. The drug was further found to decrease the incorporation of U-^{14}C-glycerol into hepatic but not intestinal triglycerides without significantly altering incorporation of ^{14}C into total phospholipids of either tissue. From these results, it appears that halofenate reduces serum triglyceride levels by inhibiting hepatic synthesis. This inhibition could occur at a site subsequent to the formation of diacylglycerophosphate from either α-glycerophosphate or dihydroxyacetone phosphate. Clofibrate at 0.25% in the diet produced a similar pattern of data in all the experiments with the 2-^{3}H, 1(3)-^{3}H, and U-^{14}C-glycerol (39). In related studies, it was established that these drugs do not decrease net triglyceride synthesis by stimulating hepatic lipase activities but reduce the availability of fatty acids for de novo triglyceride synthesis (40).

Studies on the effect of halofenate on lipo- and apolipoprotein patterns were carried out on hyperlipidemic rats (Sprague-Dawley, 400 g males) in which the hyperlipidemia was induced by feeding a semipurified diet containing 60% sucrose and 5% lard for 3 wk. This regimen produces about a threefold increase in the VLDL fraction, a 50% increase in the HDL fraction and a 40% decrease in LDL (41). After this period, the animals were fed the same diet containing 0.05% halofenate for 10 more days. Serum lipids, lipoproteins and apolipoproteins of the drug-treated rats were compared with patterns obtained from non-drug treated hyperlipidemic animals and with normolipidemic rats kept on a regular chow diet.

Under these test conditions, halofenate produced about a 60% reduction in total serum triglycerides and a 30% reduction in cholesterol levels. Figure 2 shows the concentration of VLDL constituents expressed on a plasma mg/dl basis. The dietary regimen induced a marked increase in VLDL triglyceride, cholesterol, phospholipid, and protein, and halofenate produced reductions in all four components. While the values for cholesterol and protein were normalized by the drug, triglycerides were not, and thus the composition analysis shows the triglyceride:protein ratio remained high at 12.5. It is possible that if drug treatment were continued beyond 10 days the triglyceride values would also return to baseline. Fig-

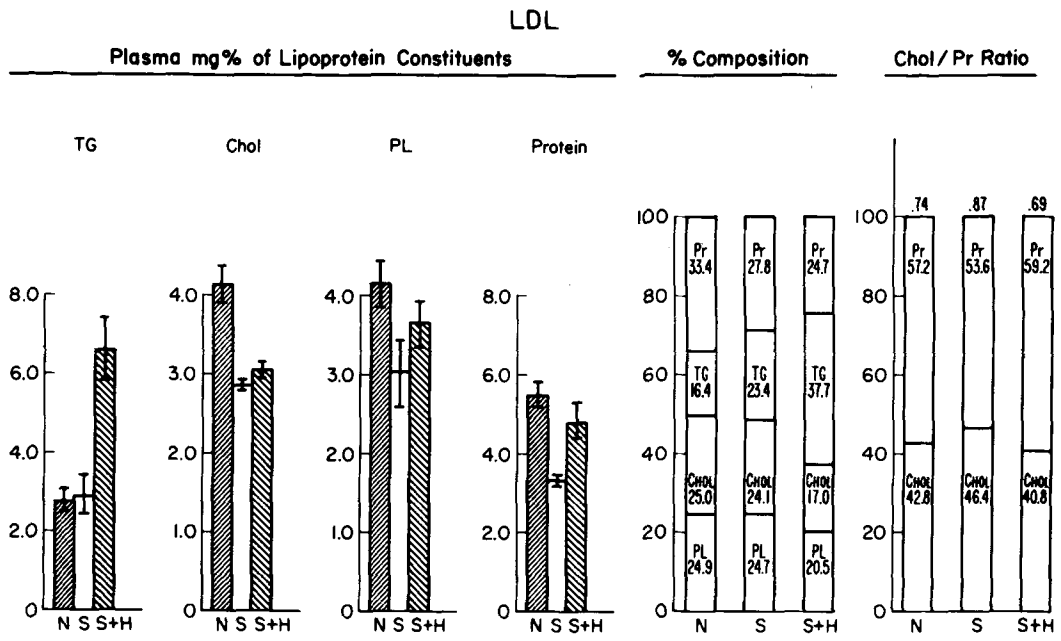


FIG. 3. The abbreviations used are: VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein; TG = triglyceride; Chol = cholesterol; PL = phospholipid, Pr = protein; N = normal diet (for rats maintained on a normal chow diet); S = sucrose fortified semi-purified diet (for rats maintained on the 60% sucrose diet); S+H = sucrose plus halofenate diet (for rats given 0.05% halofenate in the 60% sucrose diet).

ure 3 shows the data for the LDL analysis, where only small changes in the components are seen due to sucrose feeding, with and without halofenate. As only a small percent of the total plasma lipoprotein of rat serum is LDL, the changes in amounts and composition (e.g., drop in LDL protein due to sucrose which shows a tendency to normalize with halofenate, and an elevation in LDL triglyceride due to halofenate) may not be critical to the mode of action of halofenate. Figure 4 shows the pattern for the HDL analysis. Sucrose feeding induces a rise in all four components and halofenate treatment prevents this increase. The reduction in HDL cholesterol is expected as this is the major cholesterol carrying lipoprotein in the rat.

The apoprotein profiles were determined by quantitative immunoelectrophoresis described by Laurell (42). While the total serum concentration of apoprotein B is unchanged in the sucrose fed rats, its distribution between the VLDL and LDL fraction is altered (Table VIII). In the normal rat, 22% of apoprotein B is present in the VLDL fraction compared to 76% in the serum of the sucrose hyperlipidemic rat. Halofenate tends to normalize the distribution of this apoprotein, but not to baseline values, again possibly because drug treatment for 10 days was not adequate. Nevertheless, the trend of the data suggests that halofenate may correct a defect in VLDL catabolism and thus increase its rate of removal. The sucrose-induced increases in the apolipoproteins A-I, A-IV, and C-III and in the arginine-rich peptide were also prevented by halofenate; these observations are in accord with the changes seen in the serum lipoproteins. The details of the studies on the effect of halofenate on lipoprotein metabolism will be reported separately (Roheim, to be published). It might be added that, while clofibrate produced a similar pattern of data at a five times higher level (0.25% in the diet), it had a much more rapid onset of action, showing hypolipemic activity after only one day of administration, and halofenate clearly did not.

The influence of halofenate therapy on insulin and glucagon secretion was also examined. For these studies, the Zucker obese rat with genetic endogenous hyperlipemia was used. Coincident with the plasma lipid lowering effects of halofenate, the insulin to glucagon molar ratio decreased from 2.72 in the control animals to 0.96 in rats treated with the drug for 10 days (43). Following arginine stimulation, the ratio remained reduced (0.87), in contrast to the greater ratio (2.5) seen in the control animals. The halofenate induced reduction in

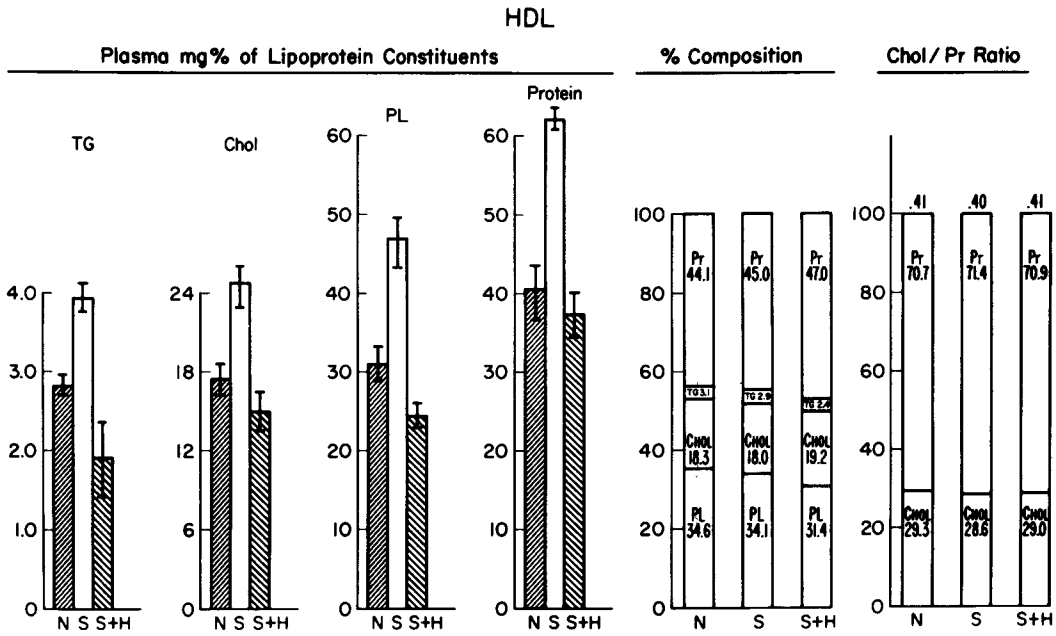


FIG. 4. The abbreviations used are: VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein; TG = triglyceride; Chol = cholesterol; PL = phospholipid, Pr = protein; N = normal diet (for rats maintained on a normal chow diet); S = sucrose fortified semi-purified diet (for rats maintained on the 60% sucrose diet); S+H = sucrose plus halofenate diet (for rats given 0.05% halofenate in the 60% sucrose diet).

the insulin to glucagon ratio was associated with hypolipemia, postarginine hyperglycemia, and hyperketonemia–metabolic parameters characteristic of glucagon excess relative to insulin. These results are similar to those seen in rats given clofibrate (44) and show that the hypolipidemic action of halofenate is associated with a reduction in the insulin to glucagon molar ratio both in the basal state and after arginine challenge. While a reduction in arginine-stimulated insulin secretion with potentiation of glucagon release has also been reported to occur with clofibrate in man (45), it would be at present inappropriate to extrapolate the data with halofenate to man.

These findings relate to the recently observed blood glucose lowering effect of halofenate in Type IV hyperlipoproteinemic patients whose pretreatment fasting blood sugar values were near 200 mg %. In this study with diabetic patients, mean blood sugar values for the group (19 patients) were decreased 25-50 mg % after 12, 24, 36, and 48 wk of halofenate treatment. About 60% of the patients had decreases of 20 mg % or greater in their individual mean fasting blood sugar values, and the mean decrease was about 80 mg % for this subset of patients. There was also an improvement in the glucose tolerance test (46; White, unpublished results). In the case of clofibrate, the changes were less consistent, with no significant drop in the mean fasting blood sugar value or improvement in glucose tolerance. Hypoglycemic activity was not observed in halofenate-treated patients where the pretreatment mean value was less than 140 mg %.

Preliminary experiments designed to study the mechanism by which halofenate reduces blood sugar have shown that this agent does not lower blood glucose in either the streptozotocin diabetic or adrenalectomized rat (Table IX). These animal models detected the hypo-

TABLE VIII

Distribution of Apoprotein B between VLDL and LDL Fractions

Animal group	Distribution (%) VLDL	LDL
Normal	22	78
Sucrose hyperlipidemic	76	24
Sucrose hyperlipidemic plus halofenate (0.05% in the diet for 10 days)	39	61

[a]VLDL = very low density lipoprotein; LDL = low density lipoprotein.

TABLE IX

Comparison of Halofenate with Other Hypoglycemic Agents

Compound	% Reduction in blood sugar: Streptozotocin diabetic rat	% Reduction in blood sugar: Adrenalectomized rat	Insulinase in vitro; % inhibition at 1 mM
Halofenate	8% (300 mg/kg p.o.)	12% (100 mg/kg p.o.)	47[a]
Clofibrate	10% (300 mg/kg p.o.)	0% (100 mg/kg p.o.)	20[a]
Phenformin	55% (200 mg/kg p.o.)	-	3
Tolbutamide	-	57% (25 mg/kg p.o.)	8
Insulin	67% (1 unit/rat s.c.)	-	-

[a]Free acid.

glycemic activity of phenformin and insulin, and tolbutamide, respectively. Moreover, when normal and streptozotocin diabetic rats were treated with halofenate at 0.05% in the diet for 21 days, there was no reduction in blood glucose (Glitzer, unpublished results). The ability of halofenate to inhibit insulinase activity in vitro (47) is only of marginal interest as it has been shown that the drug inhibits several other enzymes in vitro (see Table IV). (Mandel, unpublished results).

In summary, it is evident from this review that it is not possible to pinpoint a single enzyme or major site of action to account for the plasma hypolipidemic activity of halofenate. However, several possibilities have been explored both with isolated intact systems and in short- and long-term studies in rats. The continuing studies on hepatic triglyceride synthesis and on lipo- and apolipoprotein metabolism will undoubtedly shed more light on the mode of activity of this compound. Exploration of the effects of halofenate on plasma insulin levels both in rats and in man may offer an explanation for the hypoglycemic activity observed clinically. It should also be ponted out that there are many sites of action reported on in the published literature to explain the biological activity of clofibrate, and, though this drug has been known for a much longer period of time, the precise mechanism for its hypolipidemic effects still remains to be established unequivocally.

ACKNOWLEDGMENT

The author has been involved with the work described as a coordinator and reviewer. The contributions of Drs. W.A. Bolhofer, J.W. Huff, M.S. Glitzer, M.G. Greenspan, S.F. Kulaga, J.S. Walker and their associates of the Merck Sharp & Dohme Research Laboratories are gratefully acknowledged. I wish to thank our collaborating investigators, Drs. D. Weinstein and D. Steinberg (University of California), S.K. Erickson, M.A. Shrewsbury and R.G. Gould (Stanford University), S.A. Tepper and D. Kritchevsky (Wistar Institute), L.W. White and B.R. Landau (Case Western Reserve University), R.J. Cenedella and W.G. Crouthamel (West Virginia University), P.S. Roheim (Yeshiva University), R.P. Eaton (University of New Mexico), and their associates for their contributions and assistance.

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Gemcadiol: A New Antilipemic Drug, A Clinical Trial

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ABSTRACT

Nineteen patients with Type II, III, IV, or V hyperlipoproteinemia received gemcadiol (2,2,9,9,-tetramethyl-1,10-decanediol) in daily doses between 1.2 and 2 g. The patients were hospitalized for dose titration and then followed as outpatients on appropriate doses of gemcadiol. In Type II hyperlipoproteinemia, serum cholesterol was lowered by an average of 24%. The serum triglycerides in Type IV hyperlipoproteinemia decreased by an average of 51%. Serum uric acid was not altered. There were no changes of subjective feelings or of clinical or laboratory findings, including creatine phosphokinase. This study indicates that gemcadiol is an effective antilipemic agent that is very well tolerated.

INTRODUCTION

Gemcadiol (2,2,9,9,tetramethyl-1, 10-decanediol), Figure 1, (CI-720) is a new antilipemic agent of potential use for the treatment of patients with hypertriglyceridemia and/or hypercholesterolemia. Preclinical studies have indicated that gemcadiol reduces both serum triglycerides and cholesterol. In several animal species, the pharmacologic activity of gemcadiol is different than clofibrate, but the mechanism for reduction of serum lipids is not yet known. In view of the association of elevated serum lipids and coronary heart disease, efforts continue to develop a drug that will influence the morbidity and mortality of cardiovascular heart disease by reducing serum lipids.

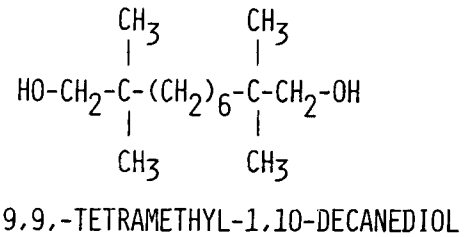

FIG. 1. The structural formula of gemcadiol.

This study describes an early clinical evaluation of gemcadiol in hyperlipemic subjects in which several dosages were administered to patients both in and outside the hospital.

METHODS

The patients participating in this study were adults of either sex ranging in age from 40 to 67 yr with elevated serum cholesterol and/or serum triglyceride concentrations. The serum cholesterol was considered to be elevated if it was greater than 250 mg/100 ml, and the serum triglyceride was elevated if it was greater than 150 mg/100 ml. Lipoprotein-phenotyping was done onall patients. The patient characteristics by lipoprotein phenotype, age, sex, race, weight, and concurrent diseases are shown in Table I. Five of the six patients with Type II hyperlipoproteinemia had evidence of ischemic heart disease, and two of these five had a history of previous myocardial infarction. Seven

TABLE I

Patient Characteristics Gemcadiol

HLP[a] Type	No. of Patients	Age	Sex	Race[b]	Weight	Concurrent diseases[c]
II	6	52 ± 9	3 F	5 B	164 ± 16	3 HBP, 2 DM (one on insulin)
II						5 IHD, 2 with myocardial infarction
III	1	52	F	B	145	IGD, PVD
IV	11	55 ± 9	7 F	8 B	191 ± 40	9 HBP, 7 DM (3 on insulin, 5 IHD)
			5 M	4 W		2 gout, 1 cerebrovascular disease, 1 PVD
V	1	42	F	W	150	Meniere's Disease

[a]HLP–hyperlipoproteinemia.

[b]B–black, W–white.

[c]HBP–hypertension; DM–diabetes mellitus; IHD–ischemic heart disease; PVD–peripheral vascular disease.

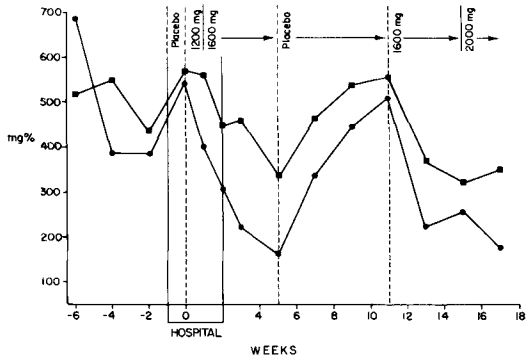

FIG. 2. The design of the study as illustrated in Patient H.J. (■ = cholesterol; ● = triglycerides)

of the eleven patients with lipoprotein Type IV abnormality had diabetes mellitus, five had ischemic heart disease but no history of myocardial infarction, and two had gout. A total of 11 of the 19 patients had some manifestation of atherosclerotic vascular disease. Of the nine diabetics, four were on insulin while others were controlled on diet alone. A dietary history was taken on each patient and the caloric intake as well as a breakdown of carbohydrate, protein, and fat content was determined. During the course of the study, however, there was no change in the patients' dietary habits.

The design of the study is illustrated in Figure 2; Patient H.J. is taken as an example. Initially there were 6 wk of pretreatment observations. The patients were followed as outpatients during the first 5 wk and were admitted to the Therapeutics Ward of Charity Hospital and stabilized on the hospital diet and activity for the final pretreatment week. During the pretreatment period, the patients received 10 mg placebo capsules in two equally divided doses daily. Active drug administration began with doses ranging from 400 to 1200 mg of gemcadiol. The total daily dose was increased by increments of 400 mg daily at 7 day intervals to a maximal dose of 2000 mg per day or until either efficacy or intolerance was encountered. Efficacy was defined as a 20% reduction of serum cholesterol and/or triglyceride concentrations as compared to pretreatment mean levels. When an effective dose was determined, this dose was maintained for a total of at least 4 wk, 1 wk of which was an inpatient period. If the lipid lowering was maintained for 4 wk period, identical placebo was substituted until the serum lipids returned to the pretreatment control values. At that point, active therapy was again reinstituted at the previously effective dose level for long-term followup.

In the absence of a satisfactory response,

TABLE II

Serum Lipids at the End of Each Treatment Period

Patient No.	Age	Race	Sex	LP Type	Cholesterol (mg/100/ml)							Triglycerides (mg/100/ml)							
					Baseline	400mg	800mg	1200mg	1600mg	2000mg	Washout	Re Rx	Baseline	400mg	800mg	1200mg	1600mg	2000mg	Washout	Re Rx
1.	34	W	M	IV	359	486	493	406	359	266	326	–	970	1265	1280	1038	549	261	368	–
2.	62	B	M	IV	176	244	220	231	220	189	208	203	192	435	303	324	443	155	411	129
3.	63	B	F	II	392	343	369	226	322	221	–	–	216	152	194	143	118	107	–	–
4.	43	B	M	II	298	303	290	300	329	255	287	–	79	119	125	159	71	95	73	–
5.	62	B	F	II	306	–	–	263	207	–	265	220	203	–	–	166	127	–	144	108
6.	42	W	M	II	302	–	–	328	313	232	307	281	203	–	–	233	186	126	153	122
7.	48	B	M	II	297	–	–	241	249	222	297	245	127	–	–	67	79	108	132	76
8.	51	B	F	II	290	–	–	305	276	245	307	–	109	–	–	85	89	95	128	–
9.	56	W	M	IV	264	–	–	256	196	–	216	–	202	–	–	145	141	–	193	–
10.	60	B	F	IV	240	–	–	218	190	–	254	237	244	–	–	186	146	–	156	167
11.	59	B	F	IV	403	–	–	339	272	–	325	209	263	–	–	154	76	–	203	72
12.	54	W	F	IV	371	–	–	323	292	–	307	246	314	–	–	220	148	–	300	122
13.	52	B	F	III	567	–	–	557	458	–	533	350	541	–	–	401	221	–	441	174
14.	67	B	M	IV	417	–	–	385	346	295	438	281	286	–	–	227	287	357	445	277
15.	60	B	M	IV	256	–	–	213	–	–	202	196	174	–	–	94	–	–	208	118
16.	49	B	F	IV	465	–	–	352	301	–	439	234	756	–	–	391	351	–	–	255
17.	57	B	F	IV	364	–	–	383	349	284	325	175	461	–	–	369	314	255	270	309
18.	42	W	F	V	478	–	–	290	225	161	254	153	2547	–	–	1260	694	385	977	312
19.	50	B	F	IV	298	–	–	246	246	206	219	187	436	–	–	265	241	149	401	182

TABLE III

Inpatient Dose–Titration: Appropriate Gemcadiol Dose vs. Pretreatment

Type	Cholesterol		Triglycerides	
	Pretreatment	Gemcadiol	Pretreatment	Gemcadiol
	(T_1)	(T_2)	(T_1)	(T_2)
II	319.7 ± 38.1	235.3 ± 36.8[a]	165.5 ± 46.8	106.3 ± 16.3[a]
III	567	447	541	308
IV	318.8 ± 77.4	281.3 ± 60.9	332.7 ± 183.9	209.9 ± 82.0[b]
V	478	179	2547	416

[a]$T_1 \neq T_2$ ($p < 0.05$) Wilcoxon Test
[b]$T_1 \neq T_2$ ($p < 0.01$) Wilcoxin Test

TABLE IV

Outpatient Phase: Gemcadiol vs. Pretreatment vs. Washout Periods of Study

Type	No. of patients	Cholesterol Changes		
		Pretreatment	Gemcadiol	Washout
		(T_1)	(T_2)	(T_3)
II	6	299.9 ± 23.4	228.7 ± 27.4[a]	286.8 ± 18.9
III	1	514	338	555
IV	11	317.7 ± 88.9	244.8 ± 50.7[b]	289.7 ± 84.3
V	1	430	190	219

Type	No. of patients	Triglyceride changes		
		Pretreatment	Gemcadiol	Washout
		(T_1)	(T_2)	(T_3)
II	6	130.0 ± 19.6	93.8 ± 21.5[c]	125.8 ± 27.0
III	1	496	163	508
IV	11	387.8 ± 250.3	189.8 ± 126.2[d]	288.8 ± 172.6
V	1	2255	997	486

[a]Treatment differ significantly ($p < 0.005$), $T_1 \neq T_2$ ($p < 0.01$), $T_2 \neq T_3$ ($p < 0.1$), Kruskal-Wallis Test followed by Dunn's Multiple Comparison.
[b]Treatments differ significantly ($p < 0.05$), $T_1 \neq T_2$ ($p < 0.1$), Kruskal-Wallis Test followed by Dunn's Multiple Comparison
[c]Treatments differ significantly ($p < 0.13$), $T_1 \neq T_2$ ($p < 0.05$), Kruskal-Wallis Test followed by Dunn's Multiple Comparison.
[d]Treatments differ significantly ($p < 0.025$), $T_1 \neq T_2$ ($p < 0.01$), Kruskal-Wallis Test followed by Dunn's Multiple Comparison.

patients were categorized as treatment failures and terminated from the study. Serum cholesterol, triglycerides, and clinical laboratory determinations (CBC, urinalysis, and SMA-12) were obtained at each outpatient visit, which was weekly to biweekly, and during the inpatient period at least weekly. Plasma drug concentrations were determined during the treatment period weekly as an inpatient and at each outpatient visit. Serum cholesterol was determined by the method of Liebermann-Burchard (1) and triglycerides by the method of Eggstein and Schmidt (2).

RESULTS

The serum lipid values at the end of each treatment period for each patient is listed in Table II. Since a portion of the study was conducted in a hospital and the remainder on an outpatient basis, the statistical analyses were performed separately in order to minimize dietary and environmental variables. Since a dose of 400 to 800 mg of gemcadiol did not reduce serum cholesterol or triglycerides in the first four patients, in the following 15 patients the starting dose was increased to 1200 mg/day.

Table III lists the mean serum cholesterol and triglyceride values during the inpatient treatment. The mean values obtained during treatment with the highest dose of gemcadiol are compared with the pretreatment control values. There was a significant decrease in the

TABLE I

Gemcadiol Clinical and Laboratory Changes (Means ± S.D.)

	Pretreatment (T_1)	Final Dose (T_2)	Washout (T_3)
Weight (lbs.)	181.7 ± 35.4[a]	176.6 ± 31.3	176.5 ± 32.1
Hemoglobin (gm%)	14.1 ± 1.1[a]	13.2 ± 1.1	13.5 ± 1.0[b]
Blood urea nitrogen (mg/100 ml)	16.0 ± 4.2[c]	18.7 ± 5.9	16.9 ± 2.3
Uric acid (mg/100 ml)	6.4 ± 1.7	6.4 ± 2.1	6.3 ± 2.3
Creatine phosphokinase (unit)	35.2 ± 29.7	48.6 ± 39.8	46.1 ± 33.8
Blood pressure Systolic (mmHg)	136.0 ± 23.1	131.5 ± 21.2	132.0 ± 10.4
Blood pressure Diastolic (mmHg)	76.9 ± 10.5	76.8 ± 11.0	78.0 ± 11.6

[a] $T_1 \neq T_2$; $T_1 \neq T_3$; $p < 0.01$ Wilcoxon Test
[b] $T_2 \neq T_3$; $p < 0.05$ Wilcoxon Test
[c] $T_1 \neq T_2$; $p < 0.02$ Wilcoxon Test

serum cholesterol and triglycerides in the Type II patients. A more highly significant reduction of the serum triglycerides in the Type IV patients occurred,and although the serum cholesterol came down, the reduction was not significant according to the Wilcoxon Test (3). Since there was only one patient with the Type III abnormality and one patient with the Type V abnormality, statistical tests were not performed, but one can see that the reductions were impressive. For the outpatient phase, there were two placebo periods: the pretreatment and washout periods. The comparative lipid values for outpatient treatment are listed in Table IV. There was a significant reduction of both the serum cholesterol and the triglycerides in the Types II and IV patients according to the Kruskal-Wallis Test (4) followed by Dunn's Multiple Comparisons (3,4). When the active and pretreatment periods were compared, there was a trend towards the pretreatment values during the washout period, but these values were not statistically significant.

The clinical and laboratory changes of greatest interest, besides the primary endpoints, are listed in Table V. There were some fluctuations in these parameters of statistical significance according to the Wilcoxon Test (2), but the only one that appears to be clinically important is the weight decrease of ca. five pounds. This was not accounted for by any forced alteration in the patient's food intake other than perhaps a greater interest on the part of the patient due to the regimentation involved in being in a study. The changes in the hemoglobin are to some degree accounted to the quantity of blood drawn during the course of the study.

The plasma levels of gemcadiol and its diacid metabolites are illustrated in Figures 3 and 4. A good dose response was obtained when the values were plotted on a mg/kg dose basis for the diacid metabolites, but a similar association was not noted for the gemcadiol plasma levels.

TABLE VI

Side Effects

Sympotoms	Treatment	Placebo	Both
Headaches	3	3	1
Weakness, tiredness	2	3	0
Dizziness, faintness	2	3	0
Chest pain	1	3	3
Depression	2	0	1
Constipation	1	1	0
Loss of appetite	1	0	0
Puffiness	1	0	0
Common cold	3	2	0
Neck and back pain	2	3	2

There were relatively few adverse effects reported during treatment with gemcadiol. These are listed in Table VI as they occurred in the treatment, placebo, or both periods. The drug appeared to be quite well tolerated in the doses employed.

DISCUSSION

Because of the association of hypercholesterolemia and hypertriglyceridemia with coronary heart disease, there are continuing efforts to develop treatments that will lower serum cholesterol and/or triglycerides with the ultimate goal of reducing the morbidity and mortality of coronary heart disease. Studies to date have shown that serum cholesterol and triglycerides can be reduced by either dietary or pharmacologic treatment. At the present time, there are relatively few drugs which will effectively and safely lower serum lipids, and there is contradictory evidence regarding the effectiveness of pharmacologically-lowered serum lipids

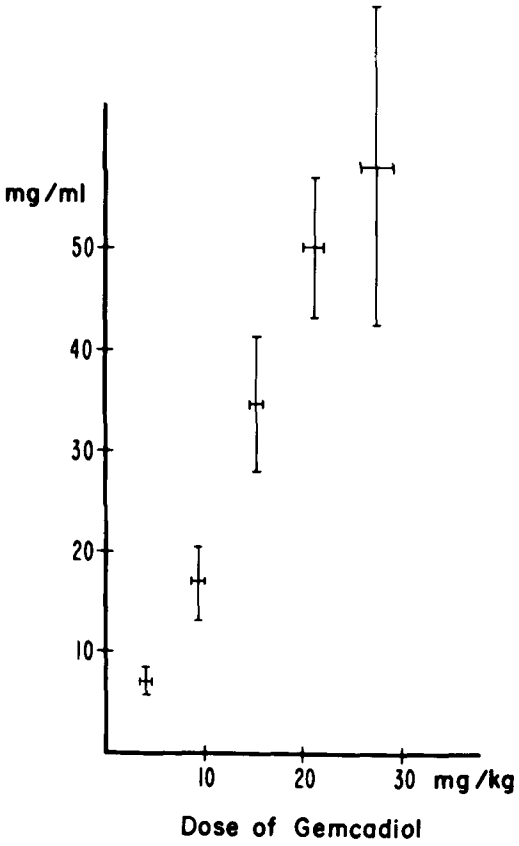

FIG. 3. The plasma levels of gemcadiol diacid metabolites (Means ± S.E.M.)

on coronary heart disease morbidity and mortality. It would appear that if preventive measures are to be effective, they would have to be instituted prior to the first myocardial infarction rather than afterward. As such, relatively long-term studies are required to demonstrate the effectiveness on morbidity and mortality.

Gemcadiol is a new antilipemic agent developed by Parke-Davis. The data reported here would suggest that the diacid metabolite rather than gemcadiol causes the reduction in serum cholesterol and triglycerides.

This study demonstrated that in doses of 1200 to 2000 mg/day, gemcadiol effectively lowers serum lipids in patients with either hypercholesterolemia or hypertriglyceridemia.

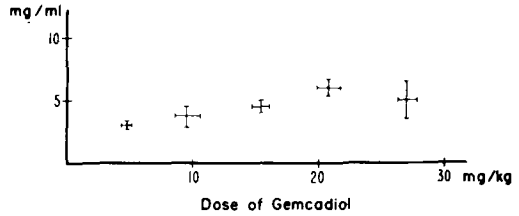

FIG. 4. The gemcadiol plasma levels (Means ± S.E.M.)

The mechanism by which serum lipids are lowered is not known at the present time and was not specifically evaluated with this study. There was a slight reduction in weight which occurred through the course of treatment. However, the magnitude of this does not appear to be sufficient to account for the degree of reduction in serum lipids. There was no anorexia, nausea, or vomiting and no change in bowel habits. Gemcadiol in fact was quite well tolerated. There was a slight reduction in hemoglobin, but this does not appear to be significant. In fact, there were no symptoms at all that appeared to occur with a greater degree of frequency during the treatment period as opposed to the placebo periods. Likewise, the laboratory parameters remained quite stable throughout the course of the study. Of particular note is that no change occurred in creatine phosphokinase, liver function (5), or uric acid (6) studies as has been reported with other antilipemic drugs.

Although experience with gemcadiol is relatively limited, it does appear to offer promise as an antilipemic agent, because it is effective and to date has been well tolerated.

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Diet and Cholesteremia

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ABSTRACT

The statistical correlation between elevated serum cholesterol levels and increased risk of coronary heart disease has channeled thinking towards regarding dietary fat and cholesterol as the principal causes of hypercholesteremia. Since 1909 there have been a number of changes in nutrient availability in the United States. Protein availability is unchanged but the ratio of animal to vegetable protein rose from 1.06 in 1909 to 2.37 in 1972. Fat availability has risen by 26% with the ratio of animal to vegetable fat falling from 4.88 in 1909 to 1.64 in 1972. A review of available data indicates that amount and type of protein may affect cholesteremia and atherosclerosis in experimental animals. Soy protein is less cholesteremic than casein but the extent of difference can be affected by the type of carbohydrate. In a semipurified, cholesterol-free diet, saturated fat exerts an atherogenic effect but unsaturated fat does not. No atherogenicity is evident when saturated fat is added to laboratory ration. In the semipurified diet, fructose is more atherogenic than glucose, and casein is more atherogenic then soy protein. The latter difference can be virtually eliminated if alfalfa replaces cellulose as the dietary fiber. The data suggest that all elements of the diet interact and can affect cholesteremia and atherosclerosis. These effects must be considered in the planning of dietary intervention trials.

The relationship between elevated serum lipid levels and the risk of developing coronary artery disease is well established. Because of the correlation of heart disease with elevated serum cholesterol levels, most attempts at dietary intervention have centered upon limitation of dietary fat in general and saturated fat and cholesterol in particular. The rationale behind such trials and some of the results have been reviewed by Dayton (1) and Frantz (2), both of whom conclude that such attempts are of value. The purpose of this exposition is to discuss other dietary components that affect serum lipid levels and deserve wider recognition.

In the wake of the large increase in heart disease in the United States in the last 60 yr, and in view of the link between heart disease and serum lipid levels, it is generally assumed that changes in dietary lipids have played a major role in the proliferation of this disease. There have been a number of changes in the American diet in the last 65 yr which have encompassed all classes of nutrients. Table I shows that the per capita availability of calories was 5% lower in 1909 than in 1972. Although protein availability was unchanged in that period, the ratio of animal to vegetable protein rose from 1.06 in 1909 to 2.37 in 1972, a remarkable increase. Carbohydrate availability fell by 23% in this period, but the ratio of starches to simple sugars fell by 60%. (This change was accompanied by a 50% fall in the

TABLE I

Nutrient Availability (per capita) in the United States[a]

Component	1909	1972	% Change
Calories	3490	3320	-5
Protein (g)	102	101	---
Animal/Vegetable	1.06	2.37	+124
Carbohydrate (g)	492	381	-23
Starch/Simple Sugar	2.15	0.89	-59
Fat (g)	125	158	+26
Animal/Vegetable	4.88	1.64	-66
Fatty acids (g)			
Saturated	50.3	55.9	+11
Oleic	51.5	63.1	+23
Linoleic	10.7	23.3	+118

[a]After refs. 3-7.

TABLE II

Influence of Fat and Protein on Cholesteremia and Atherosclerosis in Pigeons[a]

	Wheat gluten/Casein			
	Low (8%)		High (30%)	
Fat	SC[b]	Ath[c]	SC	Ath
Butter	1.00	1.39	0.89	0.69
Corn oil	1.67	1.13	1.43	1.10
Crisco	0.57	3.57	0.86	1.23
Margarine	1.60	3.25	1.18	0.67

[a]After Lofland and Clarkson (ref. 38).

[b]Serum cholesterol, mg/dl.

[c]Atherogenic index.

level of dietary fiber.) Fat availability rose by 26% between 1909 and 1972, but the ratio of animal to vegetable fat fell from 4.88 to 1.64. In other words, whereas only 20% of available fat came from vegetable sources in 1909, by 1972 38% of available fat was of vegetable origin. The ratio of oleic to linoleic acid was 4.8 in 1909 and the ratio of saturated fatty acids to oleic plus linoleic acids was 0.81. In 1972 the ratio of oleic to linoleic acid was 2.7. The increase in oleic acid availability had risen by 23% in that period and that of linoleic acid by 118%. The ratio of available saturated fatty acids to oleic plus linoleic acids in 1972 was 0.65. The foregoing data, derived from papers by Friend (3), Page and Friend (4), Scala (5), Risek et al. (6) and Gortner (7), suggest that the increase in average serum lipid levels cannot be due solely to changes in lipid availability.

In general, the level of saturation of dietary fat correlates well with the severity of experimental atherosclerosis in animals fed cholesterol plus fats of various saturation (8). One anomaly is peanut oil which, despite its relative unsaturation (iodine value, 93), is atherogenic for rats (9,10), rabbits (11), and rhesus monkeys (12). A literature survey (13) has shown it is no more cholesteremic for man than is corn oil. Randomization of peanut oil renders it much less atherogenic (14), suggesting that in certain cases the structure of a fat may be as important as its fatty acid composition.

In 1958, Lambert et al. (15) reported that saturated fat alone was atherogenic for rabbits. Our own experience with saturated fat (16,17) had shown no such effect. Collation of the available literature (18) revealed that when added to ordinary rabbit ration, saturated fat did not affect either serum lipids or atherosclerosis but when added to a semipurified diet, saturated fat was hyper β-lipoproteinemic, hyperlipemic, and atherogenic. These observations suggested that some other factor in the diet was responsible for the atherogenicity of the fat. Our experiments (19,20) and those of Moore (21) indicated that the type of fiber influenced the atherogenicity of the fat. Further experiments by ourselves (22,23) and others (24) have suggested that the mechanism of fiber action may be at the level of bile acid excretion. Portman (25) and Leveille and Sauberlich (26) had previously shown that the type of fiber in the diet affected steroid excretion. Some types of fiber such as pectin (27) or guar gum (28) lower serum cholesterol levels in man, but bran does not (29). The question of dietary fiber, now of such great interest, is far from resolution. Dietary fiber contains primarily celluloses, hemicelluloses, pectins and lignins, and the mechanism of each of these components remains to be clarified. Indeed, the analytical methods are only now beginning to yield accurate assessment of the nonnutritive components of foodstuffs (30,31).

The use of semipurified diets has permitted us to assess the role of individual carbohydrates in atherogenesis. In rabbits fed cholesterol-free diets containing 14% hydrogenated coconut oil, fructose and sucrose have been shown to be more atherogenic than glucose or lactose. In baboons (22) and Vervet monkeys (32), fructose diets result in the most severe aortic sudanophilia.

Grande (33) has summarized data from a dozen experiments in which sucrose was replaced with some source of starch in human diets. The replacements were legumes, rice, cereals, potatoes, bread, or leguminous seeds. The average number of subjects was 15, the average duration 23 days, and the average exchange of calories was 23%. In seven of the twelve experiments, there was a significant drop in cholesterol levels, but all twelve experiments showed an average cholesterol drop of 13 mg/dl. The fact that the exchanged carbohydrate also contained fiber is not to be discounted.

That animal protein per se might be athero-

TABLE III

Influence of Fiber and Protein on Experimental Atherosclerosis in Rabbits[a]

	Protein			
	Casein		Soy	
Fiber	SC[b]	Atheroma	SC	Atheroma
Cellulose	402	1.50	248	1.26
Wheat straw	375	1.03	254	0.91
Alfalfa	193	0.77	159	0.73

[a]40% sucrose; 25% protein; 15% fiber; 14% hydrogenated coconut oil; 5% salt mix; 1% vitamin mix. Fed 10 months.

[b]Serum cholesterol, mg/dl.

genic was first suggested in 1909 by Ignatowski (34). Several workers have shown that casein is more atherogenic than soy protein in rabbits (35,36) and more cholesteremic in chickens (37). Lofland and Clarkson (38) summarized their experiments in pigeons and squirrel monkeys which showed that high protein diets might be more atherogenic than low protein diets. The type of fat in the diet affected the degree of this protein effect, however (Table II).

Carroll and Hamilton (39) tested the effects of defatted proteins on cholesteremia in rabbits. The diets contained 30% protein and 1% fat. Although animal proteins were more cholesteremic than vegetable proteins, the cholesteremia was affected by the type of carbohydrate. Thus, in a diet containing dextrose, casein gave cholesterol levels of 208 mg/dl and soy protein gave levels of 70 mg/dl. Substitution of wheat starch for dextrose had no effect on cholesteremia in the casein group (196 mg/dl) but lowered the cholesterol level of the soy group to 46 mg/dl. When the carbohydrate was potato starch, the cholesterol levels of the casein and soy protein groups were 48 mg/dl and 52 mg/dl, respectively.

We (40) have fed rabbits an atherogenic, semipurified, cholesterol-free diet in which the protein was either casein or soy protein and the fiber was either cellulose, wheat straw, or alfalfa. The results (Table III) show that when cellulose was the fiber the casein diet was more atherogenic and cholesteremic than the soy diet; wheat straw reduced the atherogenicity of both diets but did not affect cholesteremia; when the fiber was alfalfa, serum cholesterol levels were 52% lower in the casein group and 36% in the soy group than when the fiber was cellulose, and atherosclerosis in the two groups was lowered comparably.

The available data suggest that all components of the diet interact, all affect cholesteremia (and probably atherosclerosis), and all must be considered in the planning of dietary intervention trials.

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Maximum Lipid Reduction by Partial Ileal Bypass: A Test of the Lipid-Atherosclerosis Hypothesis

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ABSTRACT

Atherosclerosis is the process responsible for our national epidemic of coronary heart disease. A primary and proven atherosclerotic risk factor is the plasma cholesterol concentration. Yet, proof that a reduction in plasma cholesterol level results in a reduction in the incidence or severity of atherosclerosis and atherosclerotic cardiovascular disease still requires conclusive documentation. The controversial methods of operation and inconclusive results of concluded first and second generation trials are reviewed. These data clearly demonstrate that a definitive test of the lipid hypothesis has not been reported. Evidence is presented to show that the partial ileal bypass operation most nearly fulfills the six features of an "ideal" lipid lowering trial modality: maximum effectiveness (~50% cholesterol reduction); known mechanism of action; no response "escape" or "rebound"; obligatory effect without patient cooperation; minimal side effects; and relative safety. The basic design and protocol of the National Heart and Lung Institute Program on Surgical Control of the Hyperlipidemias, a secondary intervention trial using a combination of diet therapy and partial ileal bypass surgery, are outlined.

The major cause of death in the Western world is vascular disease. The principal lethal form is atherosclerotic coronary heart disease. Atherosclerosis is the underlying pathologic process which causes more than half of the annual deaths in the United States and in other industrialized nations. For the past several decades, this disease has been responsible for our national epidemic of coronary heart disease (CHD).

Atherosclerosis is a progressive disease that can begin early in life (infancy or childhood). It may be well advanced before the manifest onset of clinical disease. It is the oldest cardiovascular disease of which we have histopathological proof. Aortic remnants in Egyptian mummies dating from the mid-sixteenth century B.C. reveal atherosclerotic lesions. Probably the first description of the gross pathology of this disease was made by Leonardo da Vinci in the sixteenth century.

Although the ultimate cause of atherosclerosis is still not known, a large body of evidence, from several scientific disciplines, has led to the formulation of the lipid (or sterol) hypothesis of atherogenesis.

In the mid-nineteenth century, cholesterol was noted to be a major constituent of the atheromatous lesion. Atherosclerosis was first produced in experimental animals in the early twentieth century by increasing the plasma cholesterol concentration. The frequent association of premature atherosclerosis with diseases commonly having elevated plasma cholesterol levels (diabetes mellitus, hypothyroidism, nephrosis, familial hypercholesterolemia) was reported. Population studies from different countries demonstrated that industrialized people had higher average plasma cholesterol levels and a greater incidence of atherosclerotic diseases than those from agrarian societies.

Positive correlations between dietary fat, plasma cholesterol level, and incidence of atherosclerosis and its complications have been well documented. In the past two decades, various prospective epidemiological studies have defined several major factors which are statistically correlated with the risk of developing atherosclerotic cardiovascular disease. Among these major CHD risk factors (age, hypertension, cigarette smoking, diabetes, family history of CHD, hypercholesterolemia), the primary independent risk factor is the plasma cholesterol level. CHD risk has been shown in several population cohorts to be proportional to an exponential function of the average plasma cholesterol concentration. Additional support for the lipid hypothesis is gained from studies which have demonstrated regression of atherosclerotic lesions following plasma cholesterol reduction: (a) in animals first placed on atherogenic regimens, (b) in animals having spontaneous atherosclerosis, and (c) in a few people undergoing serial aorto-femoral and/or coronary arteriography.

The lipid hypothesis of atherogenesis states that atherosclerosis is a disease of multiple causes in which altered lipid metabolism plays a crucial and operant role. The positive association between elevated plasma cholesterol levels

TABLE I

Features of an "Ideal" Test of the Lipid Hypothesis

A. EXPERIMENTAL DESIGN:

1. Adequate number of subjects
2. Relatively homogeneous sample population
3. Minimal effects of other major CHD risk factors (exclusion or stratification)
4. Adequate randomization procedure
5. Clearly defined end-points evaluated by blind procedures when possible
6. Minimal subject drop-out rate
7. Minmal nonadherence to therapy rate
8. Adequate length of follow-up
9. Independent data analysis
10. Acceptable total financial commitment

B. LIPID LOWERING MODALITY:

1. Maximal lipid reduction
2. Known mechanism of action
3. Continuous effect; no response escape
4. Maximal adherence rate
5. Acceptable side effects
6. Acceptable safety

and the incidence of atherosclerotic cardiovascular disease does not appear to require further proof. What does require conclusive documentation is the converse of this theorem: Does a reduction in plasma cholesterol level result in a reduction in the incidence or severity of atherosclerosis and atherosclerotic cardiovascular disease? Despite the lack of clear and convincing evidence demonstrating the beneficial effect of plasma lipid reduction in man, it has become standard medical practice to employ dietary modification, hypolipidemic drug therapy, or partial ileal bypass surgery to achieve plasma lipid reduction in hyperlipidemic patients. It is, therefore, rational that well-designed and potentially definitive clinical tests of the lipid-atherosclerosis hypothesis be carried out in order that the value, if any, of lipid-lowering therapy be ascertained.

The natural history of atherosclerotic cardiovascular disease has been arbitrarily divided into a prolonged preclinical stage having no manifestations of the disease and a clinical stage in which there are overt manifestations of the disease or its complications. Therapy directed toward altering the course of the disease has likewise been divided into primary intervention and secondary intervention therapy. Clinical trials of the lipid-atherosclerosis hypothesis are similarly divided into primary intervention and secondary intervention trials. In theory, the possible effects of plasma lipid reduction on atherosclerotic cardiovascular disease, in order of diminishing success, are: (a) complete prevention, (b) reversal or regression of lesions. (c) retardation in rate of progression, (d) cessation in progression, (e) no effect, and (f) aggravation of the condition.

There have been fortuitous clinical tests of the lipid hypothesis: e.g., a reduction in the incidence of coronary heart disease in populations subjected to prolonged under-nutrition during the second World War and an increase in CHD incidence in populations that migrated from areas of low- to areas of high-incidence of the disease (e.g. orientals migrating to the United States and Yemeni jews to Israel). These anecdotal observations, however, do not prove the lipid-atherosclerosis hypothesis.

During the past twenty years, there have been a number of clinical trials reporting the effect of plasma cholesterol reduction, by dietary or drug therapy, on atherosclerotic cardiovascular disease. These "first generation" trials have been criticized by statisticians and clinicians for certain deficiencies in design: (a) sample size inadequate to yield statistically valid results, (b) excessive age range of subjects (as young as 15 and as old as 90), (c) lack of control group, or control subjects selected from a nonrepresentational population cohort, (d) inadequate randomization procedures, (e) lack of double-blind technique in administration of treatment modality, (f) confounding effect of other CHD risk factors, (g) inadequte period of observation (high subject turnover rate), (h) significant subject dropout rate (as high as 36%), (i) possible bias in end-point evaluation (use of nonblind techniques), and (j) relatively small reductions in plasma cholesterol. Although some of the trials seemed to show a beneficial effect of plasma lipid reduction, the overall conclusion from the "first generation" clinical trials was that a definitive test of the lipid hypothesis had not been reported.

Based upon the criticisms of these "first generation" trials, as well as upon general scientific principles, ideal fetures of a test of the lipid-atherosclerosis hypothesis can be derived (Table I). Subsequent trials sought to achieve these goals.

The "second generation" clinical intervention trials began with the recently concluded Coronary Drug Project, a secondary intervention trial which showed no beneficial effect on the progression of coronary heart disease by the use of clofibrate or nicotinic acid therapy. It is important to note that the degree of plasma cholesterol reduction obtained with these two drugs was 6.5% for clofibrate and 9.9% for nicotinic acid. The Minnesota Coronary Survey, a primary intervention trial, published in abstract form only to date, also failed to show definitive beneficial effects with an average 14% plasma cholesterol reduction with diet therapy.

It would certainly be unwise, however, to conclude that the effect of plasma lipid reduction on atherosclerotic cardiovascular disease is nonexistent, on the basis of such relatively low magnitude plasma cholesterol reductions. Currently, in this country, two primary intervention trials (Multiple Risk Factor Intervention Trial and Lipid Research Clinics) and one secondary intervention trial (Program on the Surgical Control of the Hyperlipidemias) seek definitive answers to the lipid-atherosclerosis hypothesis.

At present, the three standard modes of lipid-lowering therapy are: (a) dietary modification, (b) drugs (clofibrate, cholestyramine, nicotinic acid, d-thyroxine), and (c) partial ileal bypass surgery (PIB). We have chosen partial ileal bypass as the principal lipid lowering modality for our multicentered clinical intervention trial. PIB most nearly fulfills the six features of an "ideal" lipid lowering modality (see section B, Table I):

(a) PIB is the single most effective means of lowering plasma lipids. The average cholesterol reduction is 40% below, and in addition to, the reduction obtainable by type-specific diet therapy. It is more effective than any current combination of diet and/or drugs. Figure 1 shows the average cholesterol reductions reported in several groups of the most successful studies using diet, clofibrate, cholestyramine, and nicotinic acid. These are compared to partial ileal bypass. The surgically treated patients had the greatest cholesterol reductions despite the highest pretreatment cholesterol levels. Figure 2 shows the additive effect of PIB to lipid lowering diet therapy in a group of 24 heterozygous type II hyperlipoproteinemic patients. By combining diet therapy with PIB surgery, a maximal plasma lipid reduction (~50%) can be employed in testing the lipid-atherosclerosis hypothesis.

(b) The mechanisms of action of partial ileal bypass in the treatment of hypercholesterolemia are establishment of a twofold drain on the body cholesterol pool: first, a direct drain, resulting from increased fecal loss of normally absorbed (exogenous) and reabsorbed (endogenous) cholesterol; and second, an indirect, or metabolic drain, resulting from reduced bile acid absorption. The latter process elicits increased hepatic conversion of cholesterol to its metabolic end-product—bile acids. The net result is a reduction in plasma cholesterol (and also triglyceride) concentration.

(c) The metabolic effects of PIB endure; no response "escape" or "rebound" have been observed in over 12 years' clinical experience. The procedure is reversible, although we have not found it necessary to take down a PIB.

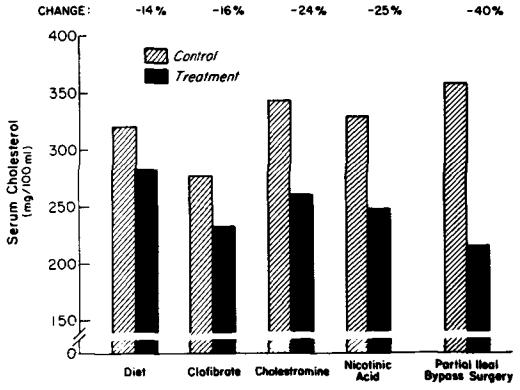

FIG. 1. Comparison of dietary, drug, and surgical treatment of hypercholesterolemia; using for the diet and drug values the means from the published reports with the maximum cholesterol lowering. (From Buchwald, H., R.B. Moore, and R.L. Varco, "Partial Ileal Bypass for Control of Hyperlipidemia and Atherosclerosis," Chapter in Advances in Experimental Medicine and Biology, Volume 63, Edited by David Kritchevsky, Rodolfo Paoletti, and William L. Holmes, Plenum Publishing Corporation, 1975, p. 221. By permission of the Editors.)

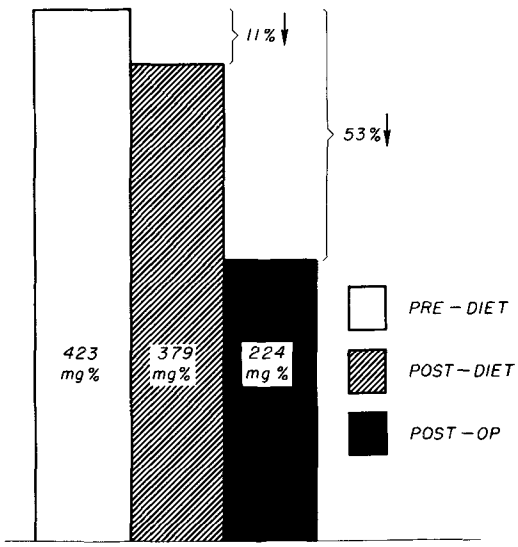

FIG. 2. Effect of cholesterol-lowering diet and partial ileal bypass on serum cholesterol levels in 24 type II patients. [From Buchwald, H., R.B. Moore, and R.L. Varco, Circulation (Suppl. XLIX) May 1974. By permission of the American Heart Association, Inc.]

(d) The effects of partial ileal bypass are obligatory and do not require continuous patient cooperation; this is an advantage in a clinical trial requiring a prolonged period of plasma lipid reduction. Figure 3 shows the

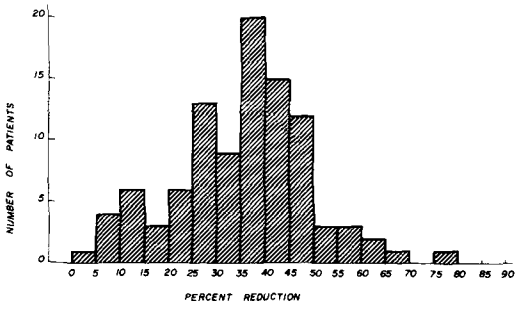

FIG. 3. Plot of the percent reduction in serum cholesterol level after partial ileal bypass; a frequency distribution bar graph in increments of 5%. [From Buchwald, H., R.B. Moore, and R.L. Varco, Circulation (Suppl. XLIX) May 1974. By permission of the American Heart Association, Inc.]

magnitude of plasma cholesterol reduction following PIB in our first 101 patients. All patients had a reduction in cholesterol level, ranging from a low of 5% to a high of 80%, with the average 40% (based on a postdietary preoperative baseline value).

(e) The side effects of this surgical procedure have been limited to transient diarrhea, which is manageable by medication, and the need for parenteral vitamin B_{12} supplementation (because of its preferential absorption site in the terminal ileum). These limitations have been acceptable in over 150 patients in our more than 13 years' experience.

(f) The operative mortality rate in our series has been less than 1%. The one death was from a myocardial infarction in a man on his fourth postoperative day. He had sustained three previous infarctions prior to his bypass.

Now, let us analyze how the features of an "ideal" experimental design (see section A, Table I) are met in our on-going trial of the lipid-atherosclerosis hypothesis.

The Program on Surgical Control of the Hyperlipidemias initiated in July 1973, is a multicentered clinical intervention trial designed to determine the effects of maximal plasma lipid reduction, using a combination of diet therapy and partial ileal bypass surgery, on atherosclerotic cardiovascular disease. This project is currently conducting a secondary intervention trial in individuals (male and female) between the ages of 30 to 59 years, who have sustained their first and only myocardial infarction within three years of entry into the trial and whose plasma cholesterol levels, while on lipid-lowering diet therapy, are greater than 220 mg/dl. The patient's myocardial infarction must be documented by specific ECG changes [using the Minnesota

TABLE II

"Second Generation" Clinical Intervention Trials

Study	No. subjects	Treatment	Effect	Appropriated funds (in millions of $)
Primary Intervention:				
Minnesota Coronary Survey (1967-1973)	9,449	Diet	-14%	6.0
Multiple Risk Factor Intervention Trial (1972 →)	12,000	Diet[a]	-10%	116.0
Lipid research clinics (1971 →)	4,000	Diet and cholestyramine	-5% plus -25% = -30%	114.0
Secondary Intervention:				
Coronary Drug Study (1966-1974)	8,341	Clofibrate	-6.5%	41.5
		Nicotinic acid	-9.9%	
Surgical control of the hyperlipidemias (1973 →)	1,000	Diet and PIB surgery	-10% plus -40% = -50%	6.8

[a]Plus other risk factor modification.

Code of Blackburn et al. (1)] and specific serum enzyme changes. We have attempted to minimize the effect of certain other CHD risk factors by: (a) excluding patients having diabetes mellitus, thyroid dysfunction, nephrosis, dysglobulinemias, or other causes of secondary hyperlipoproteinemia; (b) limiting hypertension to a diastolic blood pressure (on no therapy) of less than 105 mm Hg; (c) restricting body weight to not more than 40% over "ideal weight" (Metropolitan Life Insurance Company tables); and (d) excluding patients having aortic or myocardial aneurysm, coronary artery surgery, unstable angina, cardiomegaly, heart failure, dysrhythmia requiring drug therapy, or intraventricular conduction defects (other than RBBB and first degree A-V block) at the time of entry into the study. In addition, patients having chronic pulmonary insufficiency, chronic renal insufficiency, chronic hepatic disease, stroke or transient cerebral ischemia, malignancy, or any other condition that would make long-term survival unlikely, are excluded from the trial.

We plan to study 1,000 patients (combined total for all participating centers) who will be randomized into a control group (500) and a treatment group (500). This sample size was based on the statistical consideration that the average cholesterol reduction in the surgically treated group (40%) could lead to a reduction in mortality of 50% from that in the control group, according to the exponential risk equation developed in the Framingham study. The level of significance chosen had an alpha of .01 and the power of the test chosen was 0.99.

Upon entry into the study, but prior to the randomization process, each patient has a complete clinical evaluation which includes history, physical examination, hematology, urine analysis, blood chemistry studies, three hour oral glucose tolerance test, pulmonary function studies, chest X-rays, resting and graded treadmill exercise ECG and vectorcardiography studies, and a battery of plasma lipid tests.

During the initial phase of the study, which takes 4 to 8 wk, the patient is given information and counselling regarding the purpose, methods, and risks involved in the trial and is asked to sign the appropriate informed consent documents. The patient is then admitted to the hospital for selective coronary arteriography studies. Immediately following arteriography, the patient is randomized to either the control or the treatment group. Patients are stratified (blocked) according to (a) plasma cholesterol concentration, (b) type of hyperlipoproteinemia, and (c) extent of coronary artery atherosclerotic involvement.

Complete clinical evaluations are scheduled for each patient at 3 months, and at each year after the date of randomization for a period of at least five years. Pertinent clinical studies will be performed at any intermediate time in any patient having a significant (cardiovascular or other) clinical event. At all times, the primary care physician who has referred the patient to this study will maintain the primary medical care of the patient and he will be kept informed of all pertinent data we obtain on his patient.

Carefully defined and rigidly specified endpoints will be used to evaluate the effects of plasma lipid reduction on atherosclerotic cardiovascular disease. All electrocardiography studies are recorded on magnetic tape and are read blind in the Central ECG Laboratory using a combination of computer and human analyses. All plasma lipid tests are performed in the CDC certified Central Lipid Laboratory with no knowledge of the assigned study group of the patient. All coronary arteriograms are read in a Central Arteriography Laboratory on a blind basis. And all data analysis will be carried out by the Central Coordinating Center, under the supervision of an external Data Advisory Committee. Thus, we have attempted to remove as much investigator bias as possible in the design of this trial and to fulfill as many of the features on an "ideal" test of the lipid hypothesis outlined in Table I.

Table II lists certain pertinent features of the "second generation" clinical CHD intervention trials: (a) the number of experimental subjects, (b) the modality employed for plasma lipid reduction, (c) the actual (in the two studies which have been completed) or the projected average plasma cholesterol reductions, and (d) the total NHLI funds appropriated (through fiscal year 1977) for each study. The Surgical Control of the Hyperlipidemia Trial is projected to use the fewest subjects and to achieve the largest cholesterol reduction. The cost for the surgical trial, despite hospitalization expenditures, is 2% of the total cost for the five "second generation" trials.

It is reasonable to assume that a definitive test of the lipid-atherosclerosis hypothesis should employ a method which achieves maximal plasma lipid reduction. If maximal lipid lowering does not significantly influence the development of progression of atherosclerotic cardiovascular disease, it is unlikely that lesser degrees of lipid lowering will have a beneficial effect. The results of such a maximal lipid reduction test of the lipid-atherosclerosis hypothesis can realistically be expected to be essential to our nation. Demonstration of a significant reduction in atherosclerotic cardio-

vascular disease would, undoubtedly, stimulate more effective public health measures to alter habitual dietary patterns and provide greater incentives for the development of more effective pharmacological means of plasma lipid lowering. On the other hand, a lack of any significant alteration by maximal lipid lowering on the course of this disease strongly suggests the necessity for a redirection of scientific efforts and national resources into other areas of atherosclerotic research.

ACKNOWLEDGEMENT

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An Intervention Study—The Aspirin Myocardial Infarction Study

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ABSTRACT

The Aspirin Myocardial Infarction Study is a randomized, double-blind clinical trial designed to study the potential value of aspirin in the reduction of mortality in patients who have had a prior myocardial infarction. This trial is based upon several previously published studies which suggest that aspirin may be valuable in prevention of coronary thrombosis. The requirement of 4200 patients dictates that the study must be multicenter, and good design requires that the study be collaborative with central monitoring and an elaborate committee structure to insure uniformity and comparability.

INTRODUCTION

The Aspirin Myocardial Infarction Study (AMIS) is a multicenter, placebo-controlled, randomized, double-blind clinical trial. This trial is examining the potential value of aspirin in the reduction of three-year mortality in men and women aged 30-69 who have had a documentable myocardial infarction between 8 wk and 5 yr prior to entry into AMIS. The study began recruiting patients in June of 1975 and this phase of the study is still in progress. Thus, there are no data to present with the exception of a few figures on the recruitment of patients up to this time. However, some of the background leading to the initiation of this study will be presented, an indication of the complexity of the organization of a trial such as this will be given, some of the techniques that have been used to recruit patients into the study will be mentioned, and then the ethics of doing an aspirin study at all will be briefly discussed.

BACKGROUND

AMIS is a clinical research effort which has basic laboratory research foundation and perhaps justification, but the major stimulus for this study was several prior clinical trials of aspirin. The first of these were reported in 1953 (1) and 1956 (2) by Craven in two papers which suggested the use and the potential value of aspirin in the prevention of coronary thrombosis. However, it was not until the late 1960s that further clinical studies were initiated, perhaps because of the then clearly demonstrated antiplatelet effect of aspirin. It has now been clearly shown that ingestion of a single aspirin tablet can prevent the aggregation of a patient's platelets for as long as 1 wk. It is this action of aspirin which has been cited as the possible rationale for any effect aspirin may have in preventing cardiovascular mortality.

In 1971, Heikinheimo and Jarvinen reported on a controlled, double-blind trial of aspirin vs. placebo in 430 geriatric patients with and without a history of prior myocardial infarction (3). A double-blind clinical trial is defined as one in which neither the patient nor the medical staff knows to which treatment group the patient has been assigned. In this report by Heikinheimo, no benefit for aspirin in terms of total mortality was noted.

In 1974, Elwood and others reported on another controlled, double-blind study of aspirin vs. placebo in 1239 men all with a previous history of myocardial infarction (4). The results indicated a non-statistically significant trend in total mortality in favor of the aspirin treated group. Also in 1974, the Boston Collaborative Surveillance Group reported the results of a retrospective, case-controlled study in their hospitals (5). The results indicated that patients with a discharge diagnosis of myocardial infarction had a statistically significant ($p<0.05$) less frequent daily use of aspirin as compared to the controls, who were patients discharged from the hospital with other diagnoses.

In 1975, Hammond and Garfinkel reported results from 1,000,000 men and women fol-

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TABLE I

AMIS STRUCTURE

NHLI PROJECT OFFICE	CENTRAL LABORATORY NATIONAL CENTER FOR DISEASE CONTROL
COORDINATING CENTER UNIVERSITY OF MARYLAND	ECG READING CENTER GEORGE WASHINGTON UNIVERSITY
DRUG PROCUREMENT AND DISTRIBUTION CENTER USPHS SUPPLY SERVICE	30 CLINICAL CENTERS 18 STATES PUERTO RICO

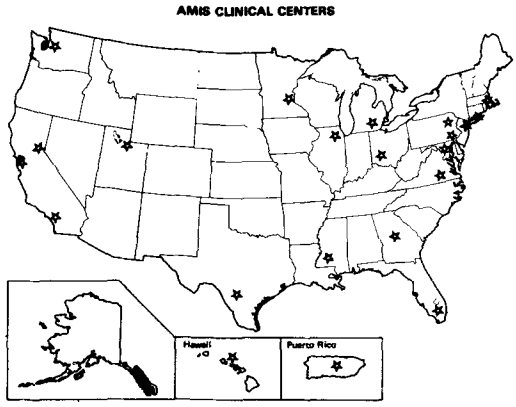

FIG. 1.

lowed for several years in a cancer epidemiological study (6). They monitored, in a prospective way, many variables including aspirin usage and cause-specific mortality. They found no significant association between reported regular aspirin usage and coronary heart disease mortality.

From 1972 to 1975, the Coronary Drug Project Aspirin Study, a randomized, double-blind study of aspirin versus placebo, was carried out in 1529 men who had previously been participants in another clinical trial and had all had a prior myocardial infarction (7). This study was supported by the National Heart and Lung Institute, and the results are currently in press. However, as a result of this study and the earlier reported ones, the Institute decided to launch a new aspirin study, the AMIS.

ORGANIZATIONAL STRUCTURE

Several major components are necessary for a collaborative clinical trial the size of AMIS (Table I). The Project Office at the National Heart and Lung Institute (NHLI) was involved in the selection process of each of the other five collaborative units and continues to work carefully with each of these in the conduct of the trial. The Coordinating Center at the University of Maryland in Baltimore has many important, pivotal functions including serving as the central unit for the collection of data from the Clinical Centers, ECG Reading Center, and Central Chemical Laboratory. These data are then transcribed onto the computer files for compilation and cross-tabulation to carefully monitor the patients for evidence of either benefit or harm from the assigned treatment regimens. The Drug Procurement and Distribution Center at Perry Point, Maryland, is responsible for ordering, labeling, and distributing the bottles of both aspirin and placebo capsules to each Clinical Center. The Drug Center also supplies acetaminophen to the Clinical Centers for distribution to each patient in the study for use as an analgesic and antipyretic. The Central Laboratory at the National Center for Disease Control in Atlanta is responsible for high quality, single center, determination of various biochemical measures on the patients in the study. The laboratory's data are transmitted to the Coordinating Center for collation with the data from other sources. The Electrocardiographic Reading Center located at George Washington University in the District of Columbia reviews all the electrocardiograms and is responsible for determining each patient's eligibility for the study (i.e., whether there is in fact electrocardiographic, laboratory, and clinical evidence for a myocardial infarction). This Center also monitors and codes the routinely obtained annual ECGs throughout the study for evidence of new cardiac events. The value of having a single center perform all the biochemical measurements and review all the electrocardiograms is that the data will be comparable from each site and tight quality control can be insured. The thirty Clinical Centers are responsible for recruiting the needed number of patients into the study and for gathering all the historical data, clinical data, and laboratory specimens on the study patients according to the standard protocol. The clinics are widely distributed throughout the continental US and are also located in Hawaii and Puerto Rico (Fig. 1).

Between October 1974 and April 1975, four Planning Committee meetings were held. This Committee was comprised of all the various participants in the trial at the time. It has as its function the transformation of the general outline of the trial as set forth in the original proposal into a detailed workable study with specific definitions, a protocol, a manual of

operations, and a set of carefully designed forms for data collection. This took an apparently long time, from October 1974 to April 1975. In fact, because of the earlier Coronary Drug Project Aspirin Study, much of the initial work had already been done. For example, initial pretested forms had been developed as a starting point for the AMIS forms. Thus, although it required 8 months from our first Planning Meeting (October 1974) until patients were first seen in the clinics (June 1975) the usual time for a collaborative study of this size has been 1-2 years.

PROTOCOL DETAILS

The patients who enter the study must be seen at a Clinical Center on at least two occasions before being accepted for enrollment and be determined to be free of any reasons for exclusion from the study such as the current use of anticoagulants, as set down in the protocol. If the patient is found to meet all of the eligibility criteria and has given written informed consent, he or she is then randomally assigned to either the aspirin or placebo group by the Coordinating Center. This is done operationally by assigning a number to each patient which corresponds to a numbered bottle prepared by the Drug Distribution Center. The aspirin (500 mgm) and placebo are prepared in identically appearing, sealed capsules with the patients instructed to take one capsule twice a day. Thus, the aspirin dosage is 1 g or the rough equivalent of three standard aspirin tablets per day. Neither the patient nor the Clinical Center staff knows whether the specifically assigned capsules contain aspirin or placebo. This information is kept at the Coordinating Center and in a sealed envelope at the local Clinical Center's Pharmacy for use in a medical emergency only. In order to help ensure that patients on placebo do not take aspirin and that patients on aspirin do not inadvertantly take extra aspirin, all pateints regardless of treatment category are given a supply of acetaminophen. The patients are instructed to use this medication at times when they would normally take aspirin.

The first AMIS patient was randomized on July 2, 1975, and, with a total patient goal of 4200 patients, it is anticipated that initial patient recruitment will end in the spring of 1976 with randomization of the last patient in the summer of 1976. With a planned follow-up of three years per patient, it is projected that the study will officially end in the summer of 1979. The patients are seen at regular 4-month intervals, and are carefully monitored for the occurrence of side effects and of various nonfatal events, cardiovascular as well as others, and for the time of death is that occurs in this high risk population. At the moment, there are eleven separate forms standardized for the collection of these data. The Coordinating Center will have stored on each patient who completes the projected follow-up period of 3 yr without an event, approximately 3100 bits of information.

TABLE II

AMIS COMMITTEE STRUCTURE

NATIONAL HEART AND LUNG INSTITUTE
POLICY-DATA MONITORING COMMITTEE
TECHNICAL GROUP
STEERING COMMITTEE

TABLE III

STEERING COMMITTEE SUBCOMMITTEES

PUBLICITY AND RECRUITMENT
ADHERENCE
QUALITY CONTROL
MORTALITY CLASSIFICATION
NON-FATAL EVENT CLASSIFICATION
ANCILLARY STUDIES
BIBLIOGRAPHY
EDITORIAL REVIEW

COMMITTEE STRUCTURE

To run a study of this size and complexity, a large number of Committees are created to oversee various aspects of the study (Table II). The NHLI is the funding agency in this contract supported study and closely interacts with each of the other units. The Policy-Data Monitoring Committee, composed of voting members who do not participate directly in the study as investigators, acts as the senior advisory committee to the NHLI and periodially reviews the study results for evidence of beneficial or adverse effects. This committee is the only one in this double-blinded study which sees the data with the treatment codes revealed. Thus, this

committee on a regular basis sees, for example, various tables and graphs of fatal and nonfatal events and side effects by treatment group. This then is the body which is directly responsible for advising that the study be prematurely stopped because of either scientific evidence of benefit or harm from aspirin administration. The Technical Group is composed of representatives from all of the operational units participating in the study and meets semiannually to review the progress of the study without, however, being privy to the blinded data. The Steering Committee provides scientific direction for the study at the operational level and is composed of representatives from each of the central units and from each of six of the thirty Clinical Centers. However, even this committee is too large and the necessary tasks too many so that to date eight subcommittees have been created to be responsible for specified aspects of the study (Table III).

The Publicity and Recruitment Subcommittee is responsible for making recommendations to the Steering Committee and Technical Group regarding methods of patient recruitment, including the use of public media.

The Adherence Subcommittee, while remaining blinded to specific drug assignments for each patient, reviews and devises methods of monitoring and maintaining good patient adherence to every aspect of the study protocol. Individual patient adherence to the assigned medication, either aspirin or nonaspirin placebo, is monitored at each clinic visit and on a random sample of the patients during the interval between visits. Two laboratory determinations are used for this purpose–(a) a urine salicylate measured only at the Central Laboratory and (b) a plasma platelet aggregation test performed at the Clinical Centers in a blinded manner and evaluated centrally. The platelet aggregation test is performed at the Clinical Centers on an aggregometer using epinephrine as the inducer, and the results are recorded directly onto a cassette tape without the use of a strip chart recorder. The tape is then mailed directly to the Central Lab for playback and reading. Thus, the personnel in the local Clinical Center are prevented from personally observing the occurrence or nonoccurrence of the second wave of platelet aggregation and from potentially unblinding a given patient's treatment assignment.

The Quality Control Subcommittee monitors the performance of each of the main operational units participating in the study and is responsible for ensuring that the data collected and stored is of high quality. The Mortality Classification Subcommittee reviews all available information concerning deaths of AMIS patients and codes the cause of death for each decedent. Thus, central blinded review of these data is guaranteed, and similar criteria can be applied to each case without the local potential of unblinding the treatment assignment with biased reporting of events. Similarly, there is a Nonfatal Event Classification Subcommittee which reviews the information on specified nonfatal events. The Ancillary Studies Subcommittee reviews proposals for additional studies within the AMIS structure, but ancillary to its main purposes. These studies are encouraged as an important fall-out from the AMIS, but they are carefully reviewed to give assurance that they will not adversely affect the primary purposes of the study and, in particular, will not interfere with the double-blind aspect. The Bibliography Subcommittee is responsible for creating and maintaining a current bibliography. And finally, the Editorial Review Subcommittee reviews and makes recommendations to the Sterring Committee on every scientific paper which comes from the AMIS, including ancillary studies. This committee is also responsible for recommending an equitable authorship policy for each publication.

PATIENT RECRUITMENT

Recruitment of patients into the AMIS has been one of the most difficult but clearly important aspects of the study. From past experience, it has been learned that recruitment into a large clinical trial such as AMIS must be approached simultaneously from several different directions. Since the AMIS patients must have all had at least one documentable myocardial infarction, the participants are almost always under the care of a physician and thus physician referral should be an important mechanism of patient contact. Unfortunately, this technique has been remarkably ineffective. Direct patient contact has proved to be the most effective mechanism after the patients have been identified by hospital chart review, coronary care unit monitoring, and direct public recruitment, using the various media including newspaper, TV, and radio. No single technique has proved to be uniformly effective in the differing Clinical Centers, thus making generalization difficult. But to date, in AMIS the two most effective mechanisms have been direct hospital chart review and newspaper publicity. Using these various mechanisms, as of April 16, 1976, 4115 patients had been seen successfully through the first visit at the Clinical Centers and 2843 patients were randomized.

ETHICS

In the Background Section, two studies were cited which supported the use of aspirin and two studies which did not. However, each of these studies has some design problems or limitations which, in our opinion, prevent the adoption of a clear position as to the value of aspirin in coronary heart disease. Despite this, one position that could be taken is that since aspirin is such a widely used, "harmless" drug, and since there is a presumed mechanism for its potential value in coronary thrombosis, i.e., antiplatelet activity, then perhaps it should be prescribed uniformly in such a high risk population as those who are recent survivors of myocardial infarction. Clearly, a major reason for not advocating this position, until the true efficacy of aspirin is determined, would be the possibility of drug toxicity. The frequency of gastrointestinal toxicity and allergic-type reactions has been reasonably well quantified in the past and is perhaps acceptable in post-myocardial infarction patients. However, the possibility of significant other toxicity, such as renal or hepatic, due to constant long term ingestion of low doses of aspirin has not been adequately investigated to be able to give assurances to patients that the chronic use of this drug is indeed safe. A study, such as AMIS, with a placebo-controlled group for comparison an a double-blind design is perhaps the only mechanism to adequately evaluate the toxicity of a drug such as aspirin.

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FDA Considerations Regarding New Hypolipidemic Agents

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ABSTRACT

Food and Drug Administration policy being considered for new marketed hypolipidemic agents includes: long-term safety to be demonstrated in postmarketing studies; evidence of clinical effectiveness to be demonstrated within a specified time period. Effectiveness is to be judged by one or more of the following: reduction in xanthomata, reduction in atherosclerotic plaque, reduction in morbidity of coronary artery disease or peripheral and cerebral atherosclerosis, and reduction in mortality. Randomized double blind trials are deemed necessary.

When Dr. Kritchevsky asked me to speak to the AOCS members on FDA's regulatory policies with respect to new antilipemic agents, my first thought was that this could be of peripheral interest only. On second thought, however, FDA's proposed policies can be expected to have a considerable impact on antilipemic drug development and, as such, would be of interest to those of you who are engaged in the synthesis and investigation of such compounds.

As you are well aware, all antilipemic drugs approved for marketing in the US were approved only on the basis of their effectiveness in lowering serum lipids with the exception that some of them were also demonstrated to cause a decrease in size of xanthomata. The antidiabetic drugs were also approved on the basis of a biochemical effect with the exception that they also ameliorated symptoms in the relatively small symptomatic patient population of maturity onset diabetics. These are the only two drug classes that come readily to mind in which drugs were approved on the basis of a hoped for, rather than a demonstrable effect on a disease.

FDA became increasingly concerned, beginning in the late 1960s, over the fact that antilipemic agents had not been demonstrated to have any impact on the associated consequences of hyperlipidemia and, thus, were being administered for chronic periods to hundreds of thousands of patients with no evidence that they might benefit. However, FDA decided to await the outcome of several secondary prevention trials then ongoing before considering any further action with respect to requiring studies for effectiveness in the prophylaxis or treatment of atherosclerosis. It did, however, convene an Ad Hoc Antilipemic Committee in 1970 which, essentially, agreed with FDA that an impact of these drugs on disease must be demonstrated but that FDA should await the results of the secondary prevention trials.

No new antilipemic agents have reached the market since then but two are nearing final action by the FDA, probucol and colestipol.

Since 1970, however, several events have transpired. First, The University Group Diabetes Program results published in 1970 and suceeding years demonstrated that a group of drugs thought to be relatively innocuous for long-term use, namely the oral hypoglycemic agents, were not as safe as believed and, in addition, there was no evidence of effectiveness on morbidity of vascular disease in maturity onset diabetics. Further, several secondary prevention studies with the antilipemic agents have failed to provide convincing evidence of any beneficial effect, and the National Heart and Lung Institute's (NHLI) Coronary Drug Project demonstrated serious deleterious though not unexpected effects of estrogen and d-thyroxine and newly discovered adverse effects with nicotinic acid and clofibrate. All of these adverse effects were of the type that could only be discovered or defined on the basis of controlled clinical trials.

FDA feels that it no longer is a viable option, consistent with protection of the public health, to approve new antilipemic agents unless long-term controlled studies are performed to demonstrate safety and, if feasible, efficacy. Such long-term studies would be performed in the postmarketing phase.

Because these studies would be supported by individual drug firms, it is essential to design a study of reasonable cost. Anything as costly as NHLI's Coronary Drug Project or its ongoing Type II hyperlipoproteinemia cholestyramine study is clearly out of the question.

Last October, after meeting with several lipid experts, FDA presented the problem to its Biometry and Epidemiology Advisory Committee. The following proposed FDA policy, applicable to both new drugs and already marketed drugs, was provided to the Committee:

1. Long-term safety must be demonstrated in postmarketing studies.

2. Evidence of clinical effectiveness for a disease process must be established within a specified time period, e.g., 10 years. Such evidence could be demonstrated in one or more of the following areas: (a) reduction in frequency or severity of xanthomata or symptoms of hypertriglyceridemia such as pancreatitis or abdominal pain, (b) reduction in atherosclerotic plaques, (c) reduction in morbidity of coronary artery disease or peripheral (including cerebral) atherosclerosis, (d) reduction in mortality.

No antilipemic agent, however, could be labeled merely for the reduction of xanthomas and abdominal pain in the absence of studies which attempted to demonstrate an effect on mortality and morbidity of atherosclerotic disease. The minor indications could be a "fall back" for some drugs if no demonstrable effect on atherosclerotic disease was forthcoming from these studies since there are patients who would benefit from receiving antilipemic drugs for symptomatic relief.

The Committee was asked to consider the aforementioned policies and provide guidance on reasonable studies that would meet the objectives.

The Committee did not, of course, have time to design a protocol but it did offer general, and certain specific, objectives to be taken into consideration in the design and in FDA's policy toward these drugs. For example, it recommended that, for both safety and effectiveness studies, patients in the investigational phases of a drug be carried over into the postmarketing phases. For demonstration of effectiveness, the Committee agreed that randomized double blind trials are necessary. It stated that, whenever possible, extrapolations should be permitted from one drug to another. For example, effectiveness data obtained from NHLI's ongoing primary prevention cholestyramine study could be extrapolated to colestipol, thus obviating the necessity for new effectiveness studies with colestipol. For drugs with no close chemical relatives, sich as probucol, no extrapolation is possible. The Committee also recommended that results of the studies would be more widely accepted in the scientific community if they were conducted by independent agents and not by the involved pharmaceutical firms.

To sum up the current situation with respect to the antilipemic drugs: (a) Clofibrate, d-thyroxine and nicotinic acid have been studied in several secondary prevention studies. Other than lipid lowering, there is no substantial evidence of effectiveness. Much information on the adverse effects of these drugs has been accumulated from these trials so that no further safety studies would appear to be necessary. New clinical trials will be required, however, to attempt to establish effectiveness. (b) Cholestyramine is undergoing an effectiveness study by NHLI. The data can also be used for safety. (c) Colestipol does not need to be utilized in effectiveness studies at this time but needs to be subjected to long-term safety studies. (d) Probucol must undergo both safety and effectiveness studies.

What impact will all this have? There was much discussion among FDA's lipid consultants on that issue. There was considerable sentiment that none of the drugs currently available are potent enough lipid-lowering agents to result in a demonstration of effectiveness on atherosclerotic morbidity or mortality. There were fears that FDA's policy of requiring long-term studies would discourage industry from the research required to develop more potent lipid lowering drugs. Nevertheless, they all agreed that the current laissez faire situation cannot continue.

Whereas industry will certainly be more cautious, we tend to feel that anyone who develops a truly potent and safe drug, which, by its very nature will capture a large portion of the antilipemic market, will not be inhibited by the necessity to perform long-term postmarketing studies.

Regulation of Fatty Acid Biosynthesis in Ehrlich Cells by Ascites Tumor Plasma Lipoproteins[1]

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ABSTRACT

Fatty acid biosynthesis in Ehrlich cells in vitro was reduced when very low density lipoproteins (VLDL) isolated from the ascites tumor plasma were added to the incubation medium. The degree of inhibition was dependent on the VLDL concentration. At the VLDL concentrations usually present in the ascites plasma, there was a 30% decrease in biosynthesis as measured by ^3H_2O incorporation into fatty acids. Analysis of the labeled fatty acids by gas liquid chromatography indicated that this decrease was due to a reduction in fatty acid de novo biosynthesis and that chain elongation actually was increased when VLDL were present. Although ascites plasma low- and high density lipoproteins also produced a concentration-dependent inhibition of fatty acid biosynthesis, their effects were much smaller than those of the VLDL. Studies employing VLDL and radioactive free fatty acids indicated that the cells took up and utilized fatty acids derived from these lipoproteins. When VLDL were present, labeled free fatty acid incorporation into cell phospholipids, cholesteryl esters, and CO_2 decreased, whereas its incorporation into the cell free fatty acid pool increased. By contrast, the cells incorporated only very small amounts of fatty acid from either low- or high density lipoproteins. This suggests that the VLDL exert their inhibitory effect on fatty acid synthesis by supplying exogenous fatty acids to the cells.

INTRODUCTION

Fatty acid synthesis in mammalian cells is decreased when free fatty acids (FFA) are added to the culture medium (1,2). A similar reduction occurs when Ehrlich ascites tumor cells are exposed to fatty acids complexed with plasma albumin during in vitro incubations (3). FFA are contained in the ascites tumor plasma and are readily taken up and utilized by the Ehrlich cells (4,5). Therefore, fatty acid synthesis in the Ehrlich cell probably is regulated to some extent by the FFA concentration of the ascites tumor plasma (6).

In addition to FFA, the Ehrlich ascites plasma contains high concentrations of lipoproteins when the tumor is grown in CBA mice (7). Very low density lipoproteins (VLDL) that are rich in triglycerides and cholesterol contain most of the lipids present in the ascites plasma (7). A hypertriglyceridemia is produced when CBA mice are inoculated with the Ehrlich ascites tumor, and the triglyceride-rich VLDL that accumulate in the ascites plasma resemble those present in the blood plasma of the host (7,8). Therefore, the ascites plasma VLDL probably are derived from the tumor bearing host. In vitro studies with ascites plasma VLDL containing tracer amounts of radioactive triglycerides suggest that these lipoproteins might serve as a source of fatty acids for the Ehrlich cells (9). Therefore, it was of interest to determine whether they, like FFA, also exert a regulatory effect on lipid synthesis in the Ehrlich cell.

MATERIALS AND METHODS

Lipoproteins and Cells

The Ehrlich ascites tumor was harvested from male CBA mice from 12 to 14 days following transplantation (6). Ascites plasma lipoprotein fractions were isolated by preparative ultracentrifugation at the following densities: VLDL, <1.006; low density lipoproteins (LDL), 1.006-1.063; and high density lipoproteins (HDL), 1.063-1.21 (7). Prior to these ultracentrifugations, the ascites plasma was centrifuged at 30,000 g for 30 min to remove any trace quantities of chylomicrons that were present (9). Each of the isolated lipoprotein fractions was washed twice by ultracentrifugation through a solution of appropriate maximum density, and the washed fractions were dialyzed for 24 hr at 4 C against 4 l of Krebs-Ringer phosphate buffer, pH 7.4. The buffer was changed at least twice during the 24 hr period. Each of these washed and dialyzed lipoprotein fractions migrated as a single band on

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electrophoresis (7). The triglyceride and cholesterol content of the isolated lipoprotein fractions was measured by an automated method (10,11).

The Ehrlich cells were freed of contaminating erythrocytes (6), and ca. 1 x 10^8 cells were incubated with labeled substrates as described in the legends to the tables and figures. The stock preparation of cells used in each experiment was counted with a hemocytometer. Aliquots of the suspension then were added by pipet to each incubation flask. In most of the experiments, the number of cells per flask was 1 x 10^8. After incubation, the contents of the flask were chilled and transferred into 20 ml of cold Krebs-Ringer buffer contained in centrifuge tubes. The cells were sedimented at 600 g for 5 min at 4 C, washed with 30 ml of fresh buffer and sedimented again (9). Cell lipids were extracted with a chloroform-methanol solution and the chloroform phase isolated (12). In some experiments, the lipids were separated by thin layer chromatography using a solvent system of hexane:diethyl ether:methanol:acetic acid (90:20:2:3) (13). After visualization of the lipids with I_2 vapor and sublimation of the I_2, the radioactivity contained in segments of the silica gel was determined (9). Segments of the gel were scraped directly into a scintillator solution containing dioxane (14), and the radioactivity was measured in a liquid scintillation spectrometer. Quenching was monitored with the external standard.

The rate of fatty acid biosynthesis was measured by the incorporation of ^3H from ^3H_2O (2 mCi/flask) into saponifiable lipids (6). In these experiments, the chloroform phase of the chloroform-methanol extract was saponified with ethanolic KOH and the saponifiable fraction isolated after extraction and subsequent acidification (6). One aliquot of the saponifiable fraction was taken for liquid scintillation counting. Other aliquots were taken for analysis of the amount of ^3H in individual fatty acids. The saponifiable lipids were methylated, and the methyl esters separated and trapped using a Hewlett-Packard Gas Liquid Chromatograph equipped with a 9:1 effluent stream splitter (3). The radioactive methyl ester fractions were collected in Teflon tubing immersed in 10 ml of a toluene:Triton X-100 scintillator solution and counted in the liquid scintillation spectrometer (6).

Collection of $^{14}CO_2$ was made using incubation flasks with removable center wells containing 0.2 ml of 2 N KOH. Radioactivity contained in the KOH solution was measured in 18 ml of a toluene:methanol scintillator solution (14).

Materials and Analyses

^3H_2O and ^{14}C-labeled substrates were obtained from either New England Nuclear (Boston, MA) or Amersham/Searle (Arlington Heights, IL). Unlabeled fatty acids and fatty acid methyl esters were purchased from NuChek Prep (Elysian, MN). The labeled fatty acids were purified by extraction into alkaline ethanol followed by acidification and reextraction into n-heptane (15). Bovine serum albumin, obtained from Miles Laboratories (Kankakee, IL), was defatted with activated charcoal (16). Fatty acids were complexed with albumin by adding dropwise a warm solution of the sodium soap to an albumin solution that was being stirred mechanically (15). The fatty acid content of these solutions was measured by titration (17), and the protein content was measured by the Lowry method (18). Gas liquid chromatography columns and packings were obtained from Applied Science Laboratories (State College, PA).

A modification of the procedure of Lee and Fritz was used to measure the long chain acyl CoA content of the cells (19). After incubation, the contents of the flasks were transferred to chilled centrifuge tubes containing 0.6 ml of 60% $HClO_4$. The material was sonified in an ice bath for 10 sec and the precipitate was collected by centrifugation (3). After suspending the precipitate in 3 ml of 15 mM dithiothreitol, the solution was adjusted to pH 11 to 12 with 6 M KOH. The solution was heated at 60 C for 15 min, acidified with $HClO_4$, and neutralized (3). CoA liberated by this hydrolysis was assayed by the method of Allred and Guy (20). The supernatant solution from the original $HClO_4$ precipitation was neutralized, and 1.5 ml was assayed for citrate content by the spectrophotometric method of Moellering and Gruber (21).

Each sample was counted in the liquid scintillation spectrometer for 10 min or, if the radioactivity content was low, until 1 x 10^4 cpm were recorded. Most of the samples contained at least 1000 cpm above background, except in those cases where very short incubation times were employed or the lipid class did not incorporate much radioactivity, i.e., free fatty acids and cholesteryl esters.

RESULTS

Inhibition of Fatty Acid Biosynthesis

As shown in Figure 1, each of the three ascites tumor plasma lipoprotein fractions inhibited fatty acid synthesis in the Ehrlich

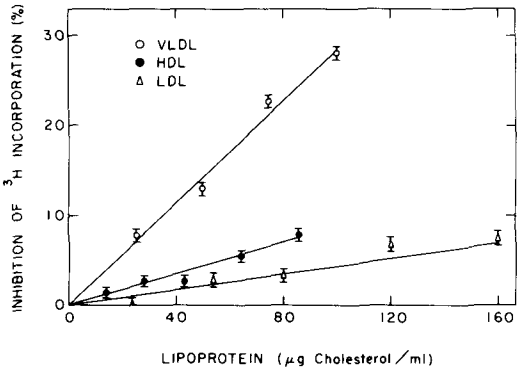

FIG. 1. Inhibition of fatty acid biosynthesis by ascites plasma lipoproteins. Incubations were for 90 min at 37 C in Krebs-Ringer bicarbonate buffer containing 11 mM glucose and 0.2 mM BSA. The gas phase was 95% O_2 and 5% CO_2. Fatty acid biosynthesis was measured with ^3H_2O as the labeled tracer. Lipoproteins were present at the concentrations indicated, based on their cholesterol content. The rates of ^3H incorporation were compared to the rate observed in control flasks to which no lipoprotein was added. Each point represents the mean of three separate cell incubations, with the vertical bars delineating the S.E. All of the data were obtained using the same preparation of cells.

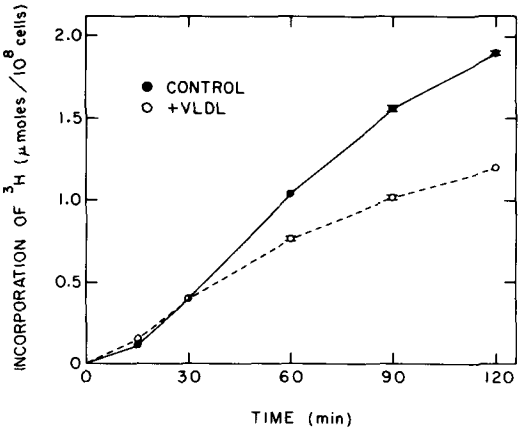

FIG. 2. The time dependence of ^3H_2O incorporation into saponifiable lipid in the presence and absence of very low density lipoproteins (VLDL). Incubations were performed as described in Figure 1 for the indicated times. VLDL were present, where indicated, at the concentration of 120 μg cholesterol/ml (860 μg triglycerides/ml). Each point represents the mean of three separate cell incubations, with S.E. delineated by vertical bars. Points without error bars had S.E. too small to be shown on the figure. All of the data were obtained using the same preparation of cells.

cells as determined by the incorporation of ^3H_2O into saponifiable lipids during a 90 min incubation. In each case, the inhibition increased as the lipoprotein concentration was raised. VLDL produced much larger reductions than either LDL or HDL. The time course of the VLDL effect is shown in Figure 2. Fatty acid synthesis was unaffected during the first 30 min of incubation. Subsequently, the degree of inhibition produced by the VLDL increased as the incubation progressed. This observation differs from that noted with FFA, where maximum inhibition of fatty acid synthesis occurs during the first 30 min of incubation (6).

In order to determine whether the inhibitory action of VLDL might be due to a nonspecific suppression of cellular metabolism, we examined the effect of VLDL on the utilization of labeled glucose by the Ehrlich cells. As shown in Table I, the oxidation of both 1-^{14}C glucose and 6-^{14}C glucose to $^{14}CO_2$ was increased slightly when VLDL were added. Moreover, the incorporation 1-^{14}C glucose into the phospholipids and triglycerides of the Ehrlich cell also was greater when VLDL were present. Similar increases in the incorporation of radioactive glucose into cell lipids occur when fatty acids are added to the incubation medium (22). Previous studies have shown that exposure to VLDL does not cause excessive lactate dehydrogenase release from the Ehrlich cells (23). Taken together, these findings indicate that VLDL do not have a nonspecific toxic effect on the cells. Therefore, like FFA, VLDL appear to specifically inhibit the fatty acid biosynthetic process.

The composite effects of VLDL plus FFA and the distribution of the ^3H incorporated into the various fatty acid fractions of the Ehrlich cells are shown in Table II. When VLDL alone were added to the incubation medium, there was a 36% reduction in ^3H incorporation into saponifiable lipids. The extent of the reduction was changed very little when laurate, palmitate, or linoleate was added in addition to the VLDL, as compared with VLDL alone. The effects of VLDL on de novo biosynthesis and chain elongation were determined by collecting five fatty acid methyl ester fractions following gas liquid chromatography. When either VLDL or VLDL together with FFA were added, there were 50% or greater reductions in ^3H incorporation into the <16:0 and 16:0 + 16:1 methyl ester fractions. Lesser reductions (7-29%) were observed in the 18:0 + 18:1 fraction. Except in the case where both VLDL and palmitate were added, increases in incorporation were observed in the 18:2-20:4 and >20:4 fractions. In the Ehrlich cell, ^3H incorporation into the <16:0 and 16:0 + 16:1 fractions occurs primarily through de novo fatty acid synthesis, except when laurate is available (3,6). Incorporation of ^3H into the 18:0 + 18:1 fraction occurs

TABLE I

Effects of Ascites Plasma VLDL on 1-^{14}C Glucose and 6-^{14}C Glucose Utilization by Ehrlich Cells[a]

Metabolite	VLDL[b]	Glucose Incorporation[c]	
		1-^{14}C Glucose	6-^{14}C Glucose
		nmoles/10^8 cells	
CO_2	None	559 ± 10	119 ± 2
	Present	608 ± 8[d]	129 ± 6
Phospholipids	None	149 ± 4	ND[e]
	Present	175 ± 3[d]	ND
Triglycerides	None	22 ± 1	ND
	Present	36 ± 1[f]	ND

[a]The incubation media contained 1 x 10^8 cells, 0.4 μmole of albumin and 21 μmoles of glucose. Incubation was for 1 hr at 37 C.

[b]The very low density lipoproteins (VLDL) added contained 1.86 μmole triglyceride and 1.32 μmole cholesterol.

[c]Each value is the mean ± SE of 6 separate cell incubations, all done with the same cell preparation.

[d]$P<0.01$.

[e]Not determined.

[f]$P<0.001$.

TABLE II

The Composite Effects of Very Low Density Lipoproteins (VLDL) and Fatty Acids on the Distribution of ^{3}H among Different Fatty Acids Synthesized by Ehrlich Cells[a]

Incubation conditions[b]	Incorporation of ^{3}H into Fatty Acids					
	Total	<16:0[c]	16:0 + 16:1	18:0 + 18:1	18:2-20:4	>20:4
	nmoles ^{3}H/10^8 cells					
Control	474	90	180	100	66	38
VLDL	300	36	78	69	72	45
VLDL + Laurate	324	45	94	78	71	36
VLDL + Palmitate	288	31	72	94	51	40
VLDL + Linoleate	297	24	80	68	77	48

[a]Incubations were for 90 min at 37 C with the conditions described in Figures 1 and 2. Saponifiable lipids from the experiments described were methylated and analyzed using gas liquid chromatography (3). Each value represents the mean of three GLC analyses of the pooled lipids from three separate incubations.

[b]The VLDL concentration was 1.4 mg/ml of triglyceride. The molar ratio of fatty acid to albumin was 1:2.

[c]Fatty acid methyl ester fractions eluted from gas liquid chromatography are abbreviated as chain length: number of double bonds. The <16:0 fraction contains all fatty acid methyl esters with retention times less than that of 16:0; the >20:4 fractions contain all those with retention times longer than 20:4.

through both de novo synthesis and chain elongation, whereas ^{3}H incorporation into the 18:2-20:4 and >20:4 fractions occurs entirely through chain elongation. These results indicate that the reduction in ^{3}H incorporation produced by VLDL is due primarily to a decrease in de novo synthesis. Moreover, they indicate that FFA, when added together with VLDL, either does not further suppress de novo synthesis or suppresses it by only a small additional amount.

Additional experiments indicated that neither the long chain fatty acyl CoA nor the citrate content of the cells changed appreciably as a result of incubation in media containing VLDL. These findings are similar to those made with palmitate and stearate complexed with bovine serum albumin (3). Although these free fatty acids reduced fatty acid de novo biosynthesis by 50-60%, they also produced very little change in the total long chain fatty acyl CoA or citrate contents of the Ehrlich cells.

Lipoprotein Effects on FFA Utilization

When VLDL containing trace amounts of radioactive triglycerides were incubated with Ehrlich cells, the cells took up and utilized some of the radioactivity. These results sug-

TABLE III

Effects of Ascites Plasma VLDL, LDL, and HDL on Palmitate Utilization by Ehrlich Cells[a]

Lipoprotein fraction added[b]	1-^{14}C Palmitate Incorporation[c]				
	CO_2	Phospholipids	Triglycerides	Cholesteryl esters	Free fatty acids
	nEq/10^8 cells				
None	82.0 ± 1.3	186 ± 0.7	108 ± 0.6	16.4 ± 0.4	9.1 ± 0.5
VLDL	40.3 ± 0.3[f]	143 ± 0.5[f]	97 ± 1.0[f]	5.7 ± 0.1[f]	19.0 ± 0.6[f]
LDL	79.0 ± 0.8	175 ± 1.5[f]	103 ± 1.1[e]	17.1 ± 1.2	7.1 ± 1.2[d]
HDL	73.7 ± 0.2	173 ± 2.5[e]	105 ± 1.6	13.3 ± 0.5[e]	8.8 ± 0.2

[a]The incubation media contained 0.84 μEq of 1-^{14}C palmitate, 0.4 μmole of albumin, 21 μmoles of glucose and 1.1 x 10^8 cells in a total volume of 4 ml. Incubation was for 1 hr at 37 C with air as the gas phase. VLDL = very low density lipoproteins, LDL = low density lipoproteins, HDL = high density lipoproteins.

[b]VLDL, LDL, and HDL added contained 1570, 124, and 55 μg/ml of triglycerides and 235, 46, and 65 μg/ml of cholesterol, respectively.

[c]Each value is the mean ± SE of four separate cell incubations.

[d]$P<0.05$. Comparisons were made against the values obtained from flasks which contained no added lipoprotein.

[e]$P<.01$.

[f]$P<.001$.

TABLE IV

Effects of Ascites Plasma Very Low Density Lipoprotein (VLDL) Concentration on Palmitate Utilization by Ehrlich Cells[a]

VLDL added[b]	1-^{14}C Palmitate Utilization				
	CO_2	Phospholipids	Triglycerides	Cholesteryl esters	Free fatty acids
μg/ml	nEq/10^8 cells				
0	44	122	41	5.5	2.5
89	42	120	40	5.0	2.6
199	38	117	42	5.0	2.9
487	36	113	48	5.0	4.5
974	30	106	53	3.5	5.5
1460	27	102	55	3.0	10.0
1950	25	96	58	2.5	15.0

[a]Each flask contained 0.87 μEq of 1-^{14}C palmitate, 0.5 μmole of albumin, 21 μmoles of glucose, and 0.9 x 10^8 cells in a total volume of 4 ml. The incubation was for 1 hr at 37 C with air as the gas phase.

[b]Expressed as μg/ml of triglycerides in the VLDL.

[c]Each value is the mean of two closely agreeing determinations.

gested that VLDL triglycerides were able to supply fatty acids to the cells (9). Because FFA also suppresses fatty acid synthesis in these cells (3,6), it was reasonable to assume that the inhibition produced by the VLDL might be due to their supplying fatty acids to the cells. Therefore, we wished to determine whether the inherent lipids contained in the VLDL actually can provide fatty acids to the cells during in vitro incubations. This was tested indirectly by examining the effect of unlabeled ascites plasma lipoproteins on the utilization of 1-^{14}C fatty acids also present in the incubation medium. If the cells obtained unlabeled fatty acids from the VLDL lipids, one would expect some reduction in the rate of utilization of the radioactive FFA. As shown in Table III, addition of ascites plasma VLDL reduced the utilization of 1-^{14}C palmitate by the Ehrlich cells, primarily by decreasing its incorporation into phospholipids and its oxidation to CO_2. By contrast, 1-^{14}C palmitate incorporation into the cell FFA pool increased when VLDL were added. Since the VLDL did not suppress glucose utilization (Table I) or cause lactate dehydrogenase release, these effects are not due to a nonspecific inhibition of cellular metabolism or toxicity. Neither LDL nor HDL had any appreciable effect on 1-^{14}C palmitate incorporation as compared with the lipoprotein-free medium.

Table IV demonstrates that the effect of VLDL on 1-^{14}C palmitate utilization was concentration dependent. As the VLDL concentra-

tion was raised, there was a progressive reduction in 1-^{14}C palmitate oxidation and incorporation into phospholipids and cholesteryl esters and a progressive increase in palmitate radioactivity present in the cell FFA pool. Surprisingly, 1-^{14}C palmitate incorporation into cell triglycerides also increased as the VLDL concentration was raised.

The time course of these VLDL effects on 1-^{14}C palmitate utilization is shown in Table V. Changes were apparent after 5 min of incubation, the earliest time point tested. In the case of 1-^{14}C palmitate incorporation into phospholipids, the percentage reduction produced by the VLDL after 5 min was almost as large as that occurring after 2 hr of incubation. The maximum percentage reduction in incorporation into phospholipids occurred after only 15 min of incubation with VLDL. In the case of oxidation to CO_2, however, VLDL produced a progressively larger percentage reduction up to 1 hr of incubation. Likewise, the percent change in 1-^{14}C palmitate incorporated into the cell FFA pool continued to increase during the first 90 min of incubation. More 1-^{14}C palmitate also was incorporated into triglycerides in this experiment when VLDL were present in the medium.

As shown in Table VI, VLDL produced similar effects on the incorporation of radioactive stearate, oleate and linoleate. In each case, less labeled FFA was utilized by the Ehrlich cells when VLDL were present. Labeled FFA oxidation to CO_2 and incorporation into phospholipids and cholesteryl esters were reduced in every case. Likewise, the amount of labeled fatty acid recovered in the cell FFA pool was considerably greater in every case when VLDL were present. By contrast, the effect of VLDL on labeled fatty acid incorporation into cell triglycerides was variable. When VLDL were present, the incorporation of radioactivity into triglycerides was less with 1-^{14}C stearate, unchanged with 1-^{14}C oleate and more with 1-^{14}C linoleate and 1-^{14}C palmitate.

DISCUSSION

Regulatory actions of plasma lipoproteins on mammalian cell lipid metabolism have been reported previously, but they have dealt almost entirely with cholesterol synthesis (24-26). One study has shown that the lipoproteins present in fetal calf serum regulate fatty acid synthesis in cultured hepatoma cells (27). This study was confined to comparisons between intact and lipoprotein-poor serum, and no experiments were done to specify which lipoproteins were predominantly responsible for the control of

TABLE V

Time Course of the Very Low Density Lipoprotein (VLDL) Effect on 1-^{14}C Palmitate Utilization during Incubation with Cells[a]

Time of incubation	1-^{14}C Palmitate Utilization[b]									
	CO_2		Phospholipids		Triglycerides		Cholesteryl esters		Free fatty acids	
	No VLDL	VLDL[c]	No VLDL	VLDL	No VLDL	VLDL	No VLDL	VLDL	No VLDL	VLDL
	nEq FFA/10^8 cells									
min										
5	1.5	1.2	14.6	11.9	2.9	2.9	0.4	0.4	9.9	14.3
15	9.2	6.2	41.9	34.8	14.4	15.9	1.9	0.9	8.6	15.1
30	27.4	19.0	81.4	70.9	34.9	41.1	4.5	2.5	9.9	21.2
60	65.3	40.7	148.3	118.4	77.5	80.5	9.6	4.2	15.2	34.0
90	87.9	56.3	204.4	159.1	105.0	116.1	11.7	5.9	13.2	38.5
120	110.4	71.2	242.1	185.0	129.0	137.0	15.2	7.3	12.4	38.6

[a]The incubation media contained 0.80 μmole of 1-^{14}C palmitate complexed with 0.4 μmole bovine plasma albumin in a total volume of 4 ml. The specific activity of the palmitate was 1240 DPM/nEq. Each flask contained 32 μmole of glucose and 1 x 10^8 cells. The incubation was performed at 37 C with air as the gas phase.

[b]Each value is the mean of two closely agreeing determinations.

[c]The VLDL added to the incubation medium contained 940 μg/ml of triglyceride and 161 μg/ml of cholesterol.

TABLE VI

Effect of Ascites Plasma Very Low Density Lipoproteins (VLDL) on the Utilization of Various Radioactive FFA by Ehrlich Cells[a]

1-^{14}C Fatty acid[b]	Fatty Acid Incorporation[c]									
	CO_2		Phospholipids		Triglycerides		Cholesteryl esters		FFA	
	No VLDL	VLDL[d]	No VLDL	VLDL	No VLDL	VLDL	No VLDL	VLDL	No VLDL	VLDL
	nEq/10^8 cells									
Palmitate	70 ± 1	40 ± 0.6[f]	164 ± 0.9	145 ± 2.1[f]	96 ± 0.3	104 ± 1.4[e]	8.9 ± 0.4	3.8 ± 0.3[f]	13 ± 0.5	41 ± 0.5[f]
Stearate	51 ± 1	32 ± 0.3[f]	154 ± 0.3	137 ± 0.3[f]	178 ± 1.7	115 ± 6.9[f]	7.2 ± 0.2	3.7 ± 0.3[f]	45 ± 0.4	107 ± 0.4[f]
Oleate	56 ± 1	32 ± 0.3[f]	148 ± 0.9	103 ± 0.6[f]	88 ± 0.6	88 ± 0.3	9.8 ± 0.3	4.4 ± 0.2[f]	3.8 ± 0.1	20 ± 0.6[f]
Linoleate	59 ± 3	35 ± 0.4[f]	164 ± 0.3	116 ± 0.9[f]	82 ± 0.2	94 ± 1.0[f]	7.7 ± 0.2	3.1 ± 0.3[f]	1.7 ± 0.3	4.2 ± 0.1[f]

[a]The incubation media contained 21 μmole of glucose and 1 x 10^8 cells in a total volume of 4 ml. Incubation was for 1 hr at 37 C.

[b]All media contained 0.4 μmole of albumin. The molar ratio of fatty acid to albumin 2.0.

[c]Each value is the mean ± SE of four separate cell incubations.

[d]The VLDL added contained 1250 μg/ml of triglyceride and 149 μg/ml of cholesterol.

[e]$P<0.01$. Comparisons with each acid were made between values obtained from media which contained VLDL and those which did not.

[f]$P<0.001$.

fatty acid biosynthesis. The present results, obtained with Ehrlich cells and the lipoproteins isolated from the ascites tumor plasma, suggest that VLDL exert a regulatory effect on fatty acid synthesis in this system. At a given concentration, the VLDL are about four times more effective than ascites plasma HDL and seven times more effective than ascites plasma LDL in reducing fatty acid biosynthesis (Fig. 1). Although the relative amounts of the three types of lipoproteins present in the ascites plasma vary with the age of the tumor, VLDL are the main lipid-containing lipoproteins at all times (7,8). For example, on the twelfth day of tumor growth, 94% of the triglyceride and 73% of the cholesterol present in the ascites plasma were recovered in the VLDL fraction (8). Taken together, these data suggest that VLDL may contribute to the control of fatty acid synthesis in the intact Ehrlich ascites tumor.

Two types of regulation of the fatty acid biosynthesis have been observed in mammalian cells. One is long-term control which involves changes in the cellular content of the lipogenic enzymes (28). The other is short-term control in which the activity of the lipogenic enzymes change without any change in their cellular content (1,2,28). FFA regulate fatty acid synthesis through the short-term mechanism in cultured cells (1,2), including Ehrlich cells (3,6). Since the inhibitory effects of VLDL and FFA are not additive (Table II) and the VLDL appear to supply the cells with fatty acids (Tables IV and VI), it is reasonable to assume that they might also inhibit fatty acid synthesis through a short-term regulatory mechanism. On the other hand, FFA produce maximal inhibition of fatty acid synthesis within 30 min (3,6), whereas the VLDL require about 60 min of incubation before an inhibitory effect is manifested (Fig. 2). This may be due to a slower uptake of fatty acids when they are derived from VLDL. For example, the build-up of labeled FFA in the cells continues during the first 90 min of exposure to VLDL (Table IV) but is completed within 5 min during exposure to a FFA-albumin complex (15). Yet, sufficient fatty acids are transferred from the VLDL to produce other effects on radioactive FFA utilization within 5 to 15 min (Table V). Therefore, the possibility that the ascites plasma lipoproteins act through a long-term control mechanism must be considered even though it is manifested within 1 hr of incubation.

Previous studies have demonstrated that the Ehrlich cells can utilize the triglycerides contained in the ascites plasma VLDL (9). Since labeled fatty acids from triglycerides are metabolized by these cells in a manner similar

to labeled FFA (9), the fatty acids released from VLDL-triglycerides probably enter the same cell FFA pools as fatty acids that are supplied as FFA. Such a mechanism would account for the trapping of more of the incoming labeled FFA in the cell FFA pools when VLDL are present (Tables III-VI). It also would account for the decreased rate of labeled FFA incorporation into phospholipids, cholesteryl esters, and CO_2 in the presence of VLDL; for the specific radioactivity of the cell FFA would be decreased by the influx of unlabeled fatty acids from the VLDL. Likewise, the increased 1-^{14}C glucose incorporation into cell lipids in the presence of VLDL (Table I) also is compatible with a utilization of fatty acids derived from the VLDL, the glucose providing the glycerol moieties needed for de novo triglyceride and phospholipid synthesis (22).

An observation that is difficult to reconcile with the above explanation is the different effects of VLDL on FFA incorporation into phospholipids and triglycerides, especially the stimulation of triglyceride synthesis under certain conditions. If all of the phospholipids and triglycerides were synthesized by the same de novo pathway, one would expect qualitatively similar effects of VLDL on radioactive FFA incorporation into both of these ester fractions. This would follow from the observations that diglycerides are used randomly by Ehrlich cells for phospholipid or triglyceride synthesis and that the 1-0-alkyl derivatives of glycerophosphatides and acylglycerols also are synthesized from an alkyl acyl intermediate without selectivity (29). Therefore, the differences in the effects of VLDL on labeled FFA incorporation into phospholipids and triglycerides probably reflect additional pathways for synthesis of one or both of these lipid esters. One possibility is that in the presence of VLDL some triglyceride synthesis may occur directly from lower glyceride intermediates. When VLDL containing glycerol-labeled glycerides were incubated with Ehrlich cells, glycerol-labeled glycerides were recovered in the cells (9). Since free glycerol is utilized very poorly by Ehrlich cells (9), this findings suggests the existence of a pathway in which monoglycerides are used for triglyceride synthesis (30-32), the monoglycerides being derived from partial hydrolysis of VLDL-triglycerides. This could explain the increase in radioactive FFA incorporation into triglycerides noted in some cases, particularly when the VLDL concentration was raised (Table IV), for the greater availability of monoglyceride substrate may stimulate the incorporation of certain fatty acids into triglycerides. Another possibility is that some of the fatty acid incorporation into glycerophosphatides occurs by the acylation of lysophosphatides (33), for an acylation pathway has been demonstrated in the Landschütz ascites tumor (34).

It remains to be demonstrated whether other forms of VLDL also regulate fatty acid synthesis. Although the Ehrlich ascites plasma VLDL resemble VLDL from other species in a number of ways, some differences in their chemical composition and physical properties have been observed (7). For example, the ascites plasma VLDL have a higher free to esterified cholesterol ratio, more phospholipids, increased electrophoretic mobility and a different apolipoprotein composition than human VLDL. As compared with the usual lipid concentrations present in the plasma of many species, the Ehrlich ascites plasma can be characterized as being hypertriglyceridemic (8). In this regard, it should be noted that lipoproteins obtained from hyperlipidemic rabbit plasma are much more effective in transferring lipids to cultured hepatoma cells than those isolated from normal plasma (35). Since the mechanism of suppression of fatty acid synthesis is thought to be transfer of fatty acids from the VLDL to the Ehrlich cells, it is possible that this action is dependent on the fact that these are "abnormal" VLDL and that other forms of VLDL might not produce this effect.

ACKNOWLEDGMENTS

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2-Aminoethylphosphonic Acid Metabolism in the Rat

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ABSTRACT

Time course studies of the incorporation of radioactive 2-aminoethylphosphonic acid (AEP) into the tissues of rats demonstrated that maximum incorporation into the liver lipids occurred within 12 to 30 hr after injection, compared to 2 to 3 hr for the incorporation of phosphorylethanolamine. Little incorporation of AEP was observed in the other tissues investigated (heart, lung, spleen, adipose, kidney). The AEP was incorporated to the greatest extent into 1,2-diacylglyceryl-aminoethylphosphonate (diacylglyceryl-AEP), the phosphonate analogue of phosphatidylethanolamine, with some incorporation into the lyso derivative. Diacylglycerol-AEP apparently was not further metabolized by the rat; no methylation of diacylglyceryl-AEP to phosphonolecithin was observed. Subcellular fractionation was performed on the livers of rats who received ^{3}H-AEP 12, 30, 36, and 48 hr prior to sacrifice. The greatest amount of radioactivity was recovered in the soluble fractions. Lipid extraction was performed on the subcellular fractions, and most of the radioactivity present in the lipids was found in the microsomal fraction, with the next highest recovery in the mitochondrial and nuclear fractions.

INTRODUCTION

In 1959, 2-aminoethylphosphonic acid (AEP) was isolated from the ciliated protozoa of sheep rumen (1). Since then, AEP and related compounds have been isolated in the free form, bound to lipid, and bound to protein in a variety of organisms, including protozoa, marine invertebrates, and fresh water mollusks (see References 2 and 3 for thorough reviews through 1969).

Most recently, AEP has been isolated from mammalian tissues. In 1965, it was isolated from bovine brain (4) and goat liver (5). Since then AEP has been found in human brain, liver, heart, and skeletal muscle (6,7). N-Trimethyl-2-aminoethylphosphonic acid (cholinephosphonic acid) has been isolated from atheroschlerotic plaques of human aorta (8). Although it has been determined that mammals are incapable of de novo synthesis of AEP (9), alkylphosphonates are nevertheless found in significant amounts in a variety of foodstuffs commonly ingested by humans [e.g., clams, oysters, and ruminant tissues (2,3)]. For this reason, we were interested in studying the metabolism of alkylphosphonates in monogastrates.

Many studies on the metabolism of AEP have been carried out on the protozoa *Tetrahymena pyriformis*. It has been determined that AEP is incorporated by *T. pyriformis* into 1,2-diacylglyceryl-aminoethylphosphonate (diacylglyceryl-AEP), the phosphonate analogue of phosphatidylethanolamine (10). In vivo and in vitro experiments demonstrated the presence of CMP-AEP in *T. pyriformis*, and it was shown by in vitro experiments that chemically synthesized CMP-AEP can transfer its AEP moiety to a diglyceride to form diacylglyceryl-AEP (11). These data suggest that AEP may be incorporated into lipids by the "Kennedy pathway" involving a CMP derivative (12).

Our laboratory previously reported (13) the results of incorporation studies utilizing [$^{14}C_{1,2}$] AEP. Approximately 16% of the labeled material administered was recovered in the rat liver lipids. Essentially all of the radioactivity in the lipid fraction was in one compound, diacylglyceryl-AEP, the phosphonate analogue of phosphatidylethanolamine. More recently, ^{32}P-AEP was found to be incorporated into rat liver lipids, with 89.3% of the incorporated radioactivity recovered in diacylglyceryl-AEP (14).

Observations have been reported which suggest that alkylphosphonates are incorporated into mammalian lipids by a mechanism involving a CMP derivative. Bjerve (15) demonstrated that the incorporation of cholinephosphonic acid into phosphonolipids was dependent upon the addition of CTP. This incorporation was inhibited by the addition of CDP-choline, indicating that CMP-cholinephosphonic acid is, perhaps, an intermediate in the synthesis of phosphonolecithin. It also was determined that

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[1]This work is based upon a thesis submitted by J.C.-J. to the Graduate College of the University of Illinois at the Medical Center in partial fulfillment of the requirements for the Ph.D. degree in Biological Chemistry. Present Address: Department of Biochemistry, Northwestern University Medical Center, Chicago, Illinois, 60611.

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cholinephosphonic acid competitively inhibited the conversion of phosphorylcholine and CTP to CDP-choline, and conversely, that phosphorylcholine was a competitive inhibitor when cholinephosphonic acid was the substrate for cytidyltransferase (15). Recent observations by Tamari et al. (16) indicated that ^{32}P-AEP was incorporated in vitro and in vivo into rat liver lipids by way of a CMP intermediate.

MATERIALS AND METHODS

Chemicals

The following materials were purchased from Sigma Chemical Company, St. Louis, MO: ethanolamine, phosphorylethanolamine, phospholipase D (from cabbage, Type I), phospholipase A_2 (*Ancistrodon piscivorus* venom), Dowex 50, Dowex 1, alkaline phosphatase (Type III), Folin and Ciocalteu's Phenol Reagent, bovine serum albumin, cytochrome oxidase (from horse heart, Type III), NADPH (Type II), NAD (from yeast, Grade V), P-nitrophenyl-N-acetyl-α-D-glucosaminide (Grade III), $MgCl_2$, ATP, and 3-phosphoglyceric acid (3-PGA). Nonradioactive AEP was purchased from Calbiochem, Oak Grove Village, IL. Glycerol-3-phosphorylethanolamine was purchased from Supelco, Bellefonte, PA. [$^{14}C_{1,2}$] Ethanolamine was purchased from New England Nuclear, Boston, MA. All remaining chemicals and solvents employed were reagent grade and were purchased from Fisher or Mallinckrodt Laboratories.

Chromatography

Thin layer chromatography was carried out on commercially prepared Silica Gel G analytical plates (E. Merck, Rahway, NJ). The solvent systems employed were:

solvent I: isopropanol/acetone/58% NH_4OH/H_2O (5:2:4:3 v/v) (17);
solvent II: $CHCl_3$/HOAc/MeOH/H_2O (75:25:5:1.8 v/v) (18).

Solvent I separates AEP from ethanolamine, and solvent II separates phosphatidylethanolamine (PE) from diacylglyceryl-AEP.

Paper chromatography was carried out on Whatman #1 chromatographic paper. The solvent systems employed were the following:

solvent III: $CHCl_3$/MeOH/17% NH_3 (2:2:1 v/v) (19);
solvent IV: MeOH/pyridine/H_2O (17:1:5 v/v) (6);
solvent V: phenol/H_2O (4:1 v/v) (16) (20);
solvent VI: 2-propanol/formic acid/H_2O (2:2:1 v/v) (9);
solvent VII: pyridine/acetone/3N NH_3 (50:30:25 v/v) (2).

All of the above systems separate AEP from ethanolamine.

Ninhydrin was used to visualize compounds containing free amine groups, while lipids were visualized with iodine vapors. Thin layer plates and paper strips were scanned for radioactivity on a Packard Model 7201 Radiochromatogram Scanner. In appropriate situations, radioactivity was also determined by scraping areas of silica gel or cutting areas of the paper strips into vials for scintillation counting in a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3310.

Synthesis and Purification of AEP and Phosphorylethanolamine

A portion of the [$^{14}C_{1,2}$] AEP employed in these studies was received as a gift from Dr. John H. Law, Department of Biochemistry, University of Chicago. The remaining portion was synthesized according to the procedure of Kosolopoff (21) as modified by Smith and Law (20).

The reaction mixture containing synthesized ^{14}C-AEP was placed on a Dowex 50(H^+) column (2.5 cm x 60 cm). Five hundred ml of H_2O, 500 ml of 20% NH_4OH, and 500 ml of concentrated NH_4OH were run successively through the column. Ten-ml samples were collected, and the radioactivity of each sample determined by liquid scintillation spectrometry. Aliquots of the samples were chromatographed on thin layer plates in chromatographic solvent I, with authentic AEP used as a standard.

The tubes containing AEP were combined, dried, and redissolved in a known amount of H_2O then applied to Dowex 1 (acetate) column (2.5 cm x 35 cm). The column was eluted successively with 500 ml of H_2O and 500 ml of 5% acetate acid, 10 ml fractions collected, and the radioactivity of each fraction determined. One large peak of radioactivity was seen, and the material was identified as AEP upon chromatography in solvent I. The AEP-containing tubes were dried down and purified by paper chromatograph utilizing chromatographic solvent III through VII.

The [G-^{3}H] AEP (G represents generally labelled), prepared for us by New England Nuclear from nonradioactive AEP, was put through a Dowex 50(H^+) column as described above, and one peak of radioactivity was seen. The ^{3}H-AEP was purified using chromatographic solvents III and IV, and the final product cochromatographed with authentic AEP in solvent I.

The phosphorylethanolamine employed in these studies was synthesized from ^{14}C-ethanolamine according to the procedure of Schneider (22). The reaction mixture contain-

ing the synthesized [$^{14}C_{1,2}$] phosphorylethanolamine was placed on a Dowex-1 (formate) column (2.5 x 60 cm). Two hundred and fifty ml of H_2O and 250 ml of a continuous 0 to .046N formate gradient were run successively through the column. Ten-ml samples were collected and the radioactivity of the samples determined by liquid scintillation spectrometer. Aliquots of the samples were chromatographed on thin layer plates in chromatographic solvent I vs. ethanolamine and phosphorylethanolamine. The tubes containing [$^{14}C_{1,2}$] phosphorylethanolamine were combined, dried, and redissolved in a known amount of H_2O, and rechromatographed on Dowex-1 (formate). The final product cochromatographed with authentic phosphorylethanolamine in solvent I.

Treatment of Animals

Young adult male rats, Sprague Dawley strain, weighing ca. 250 g, were utilized in all studies. The animals were fasted for 12-18 hr prior to injection. Each animal was then injected intraperitoneally with either [$^{14}C_{1,2}$] AEP, [G-^{3}H] AEP, or [$^{14}C_{1,2}$] phosphorylethanolamine in sterile 0.9% NaCl. The animals were allowed to eat ad libitum, beginning 1 hr after injection. The animals for time course studies were decapitated and bled thoroughly into heparinized beakers at designated times, the tissues removed, rinsed in cold 0.9% NaCl solution, and frozen until used. The animals in the subcellular localization studies were fasted for ca. 12 hr before sacrifice. At designated times, the rats were sacrificed, the liver removed, cut up, and homogenized as described below.

Subcellular Fractionation

The livers of [G-^{3}H] AEP injected rats were fractionated according to the procedure of De Duve et al. (23). The activities of various marker enzymes were estimated after freezing in a dry ice-acetone bath and thawing the subcellular fractions three times in order to release the enzymes. The activity of cytochrome oxidase (24), β-N-acetylglucosaminidase (25), NADPH cytochrome c reductase (26), and catalase (27) were determined on all fractions. The DNA (28) and protein (29) content were also determined. Lipids were extracted from the fractions by the single phase extraction procedure of Bligh and Dyer (30), and aliquots of the whole fractions and the lipid fractions were assayed for radioactivity.

Lipid Extraction

Lipid extractions of rat tissues were performed in the following manner. The tissues were individually homogenized in $CHCl_3$/MeOH (2:1 v/v) and stirred for ca. 16 hr. The insoluble residues were further extracted for 24 hr in $CHCl_3$/MeOH (1:1 v/v). The ratio of solvent to tissue was always greater than 25:1 (w/v). The 2:1 and 1:1 lipid extracts were washed with 0.1 M KCl (31), combined, dried, dissolved in 10 ml of benzene, and assayed for radioactivity. One hundred-μl blood samples and 100 mg samples of lipid-free residues were solubilized for ca. 20 hr at 55 C in 2 ml Protosol tissue solubilizer (New England Nuclear), Aquasol sintillation fluid added, and the samples counted after the vials were refrigerated for at least 8 hr. Quenching of the samples was corrected as described elsewhere (13).

Hydrolysis of Fractions

Phospholipase A_2 and phospholipase D hydrolysis of lipids were performed according to the procedures of Hildebrand and Law (32) and Yang et al. (33), respectively. The fractions were dried and dissolved in 1 ml of the appropriate solvent. Acid hydrolysis was performed on various samples with 6 N HCl for 8 hr in an autoclave (20), the hydrolysates washed four times with 5 ml of $CHCl_3$, dried, and dissolved in 1 ml of H_2O. Aliquots of the various hydrolysates were chromatographed vs. standard compounds in the appropriate solvent systems to determine the products and extent of hydrolysis.

RESULTS

^{14}C-AEP Time Course Studies

Each of fourteen rats, previously starved for 12-18 hr, was injected intraperitoneally with [$^{14}C_{1,2}$] AEP (3.3 x 10^5 dpm; specific activity: 9.1 x 10^5 dpm/μmole), which had been synthesized as described in Materials and Methods. At each of seven designated time points within a 72 hr period, two ^{14}C-AEP injected rats were sacrificed, bled thoroughly, the tissues removed and rinsed, lipids extracted and the radioactivity of the fractions determined as described in Materials and Methods. It was found that there was less than 1% incorporation into the lipids and residues from kidneys, lung, spleen, heart and adipose tissue. The results after 72 hr are shown in Table I. The level of radioactivity recovered in the tissue fractions at the other time points studied was similar.

A relatively large amount of radioactivity was recovered in the liver lipid and lipid-free residue fractions and in the whole blood. The incorporation of ^{14}C-AEP into the liver lipids reached a maximum at 24 hr (Fig. 1), at which time an average of 9% of the total injected

TABLE I

Extent of ^{14}C-aminoethylphosphonic Acid Incorporation into Rat Tissues 72 hr after Injection

Organ[a]	Amount of ^{14}C-AEP incorporated		
	Total dpm (± SEM)[b]	dpm/g tissue (± SEM)	% (± SEM)[c]
Heart			
lipid (2)	910 ± 340	1460 ± 560	0.3 ± 0.1
residue (2)	60 ± 10	95 ± 15	<0.10
Lung			
lipid (2)	310 ± 180	290 ± 150	0.1 ± 0.05
residue (2)	960 ± 200	950 ± 270	0.3 ± 0.05
Spleen			
lipid (2)	1970 ± 960	4120 ± 1720	0.5 ± 0.3
residue (2)	60 ± 1	130 ± 10	0.2 ± 0
Adipose			
lipid (2)	0	0	<0.10
residue (2)	60 ± 15	320 ± 70	<0.10
Kidney			
lipid (1)	2170	1550	0.6
residue (2)	730 ± 30	480 ± 60	0.2
Liver			
lipid (2)	25,000 ± 670	2510 ± 55	7.0 ± 0.15
residue (2)	15,000 ± 530	1520 ± 45	4.2 ± 0.15

[a]The number in parenthesis indicates the number of animals examined.

[b]SEM refers to the standard error of the mean; dpm = disintegrations per minute

[c]% refers to the percentage of total administered ^{14}C-AEP recovered in a given fraction.

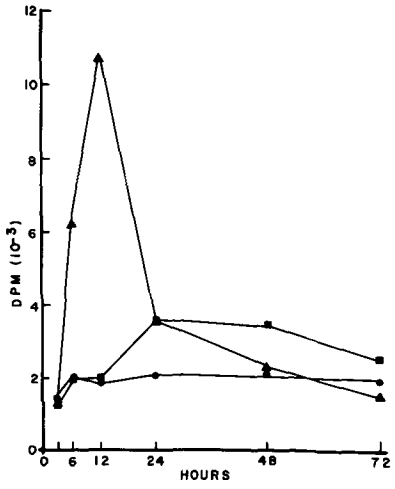

FIG. 1. Extent of incorporation of $^{14}C_{1,2}$] aminoethylphosphonic acid. Rats, previously starved for 12-18 hr, were injected intraperitoneally with [$^{14}C_{1,2}$] AEP (3.3 x 10^5 dpm; specific activity; 9.1 x 10^5 dpm/μmole). Two rats were used for each time point. The values plotted are the dpm/g liver and the dpm/ml of blood vs. the time after injection with ^{14}C-AEP. The liver lipids are shown by (■), the liver lipid-free residues by (▲), and the whole blood by (●).

radioactivity was recovered in the whole livers. The maximum incorporation of ^{14}C-AEP into liver residues occurred by 12 hr, where ca. 25% of the injected radioactivity was recovered, while the radioactivity found in the whole blood samples remained about the same throughout the 72 hr period.

^{3}H-AEP Time Course Studies

A more detailed time course experiment was next performed utilizing [G-^{3}H] AEP. ^{3}H-AEP (9.4×10^7 dpm; specific activity: 5.0×10^8 dpm/μmole) was injected intraperitoneally into each of 63 rats which had been previously starved. For the first 10 times points, five rats were employed, while seven rats were used for the 2 wk time point, and three rats each for the 1 month and 3 month time points. At each time point, the rats were sacrificed, livers removed and rinsed, the lipids extracted and the fractions' radioactivity determined as described in Materials and Methods. Maximum incorporation of ^{3}H-AEP into the liver lipids occurred by 30 hr, where ca. 16% of the injected radioactivity was recovered inthe whole livers. Maximum radioactivity was recovered in the liver residues after 18 hr, and the radioactivity present in the blood was flound to remain relatively the same throughout the time period studied, although there appeared to be an overall slight increase in incorporation after 3 months (Fig. 2).

Samples of the liver lipids were run vs. standards on silica gel plates in solvent II, and the

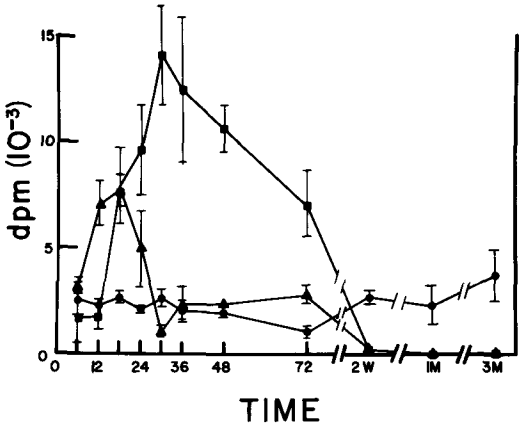

FIG. 2. Extent of incorporation of [G-^{3}H] aminoethylphosphonic acid. ^{3}H-AEP (9.4 x 10^7 dpm; specific activity; 5.0 x 10^8 dpm/μmole) was injected intraperitoneally into 63 rats which previously has been starved. For the first 10 time points five rats were employed, while seven rats were used for the 2 wk time point, and three rats each for the 1 month and 3 month time points. The values plotted are the dpm/g liver and the dpm/ml blood vs. the time after injection with ^{3}H-AEP. The symbols are (■), liver lipids; (▲), liver lipid-free residues; and (●), whole blood. The standard error of the mean is plotted for each time point. The time is given in hours except for 2W, 2 wk; and 1M and 3M, 1 month and 3 months, respectively.

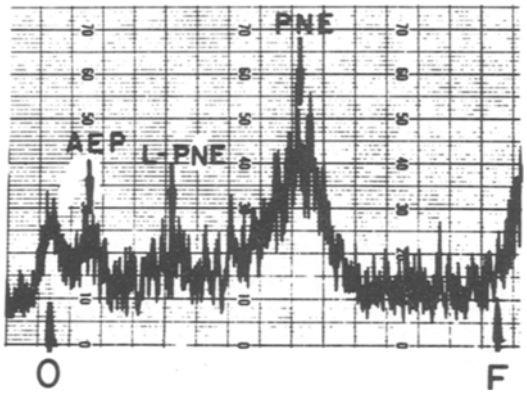

FIG. 3. Radiochromatogram of rat liver lipids 30 hr after injection with [G-^{3}H] aminoethylphosphonic acid (9.4 x 10^7 dpm; specific activity; 5.0 x 10^8 dpm/μmole). Liver lipids were chromatographed in solvent III, $CHCl_3$/HOAc/MeOH/H_2O (75:25:5:1.8 v/v) (18) vs. standard compounds. O and F indicate the origin and solvent front of the chromatogram, respectively. The radiochromatograms of rat liver lipids at the other time points were similar. PNE refers to diacylglyceryl-AEP, while L-PNE refers to the lyso derivative, monoacylglyceryl-AEP. The radioactivity to the right of the solvent front arose from a radioactive dye spot added to the plate after development to aid in registering the plate with the radiochromatographic strip recording.

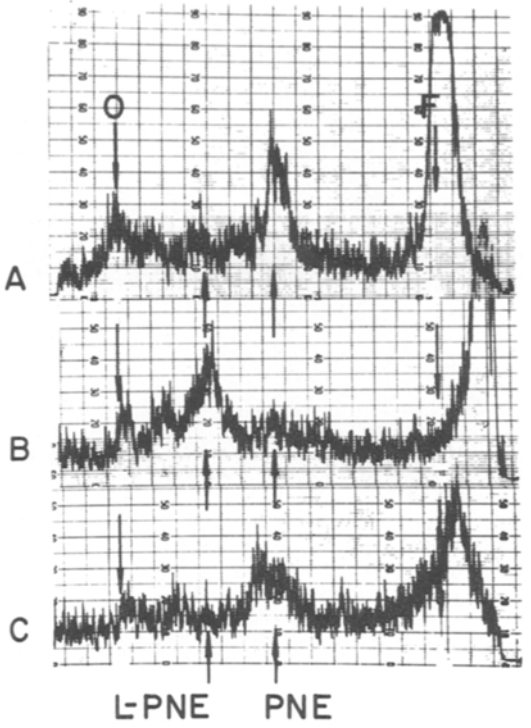

FIG. 4. Radiochromatograms of enzymatic hydrolysates of liver lipids of rats sacrificed 30 hr after injection with [G-^{3}H] aminoethylphosphonic acid (9.4 x 10^7 dpm; specific activity: 5.0 x 10^8 dpm/μmole):

(A) control plate (no enzyme treatment),
(B) liver lipids after phospholipase A_2 treatment,
(C) liver lipids after phospholipase D treatment.

The lipid samples were chromatographed in solvent III, $CHCl_3$/HOAc/MeOH/H_2O (75:25:5:1.8 v/v) (18) vs. standard compounds. O and F indicate the origin and solvent front, respectively, while PNE and L-PNE indicate diacylglyceryl-AEP and monoacylglyceryl-AEP, respectively. Similar radiochromatograms were obtained at all time points studied. The radioactivity at, or beyond the front arose from a radioactive dye spot as described for Figure 3.

plates were scanned for radioactivity on a radiochromatogram scanner. A representative strip scanning is shown in Figure 3. Four peaks of radioactivity were detected. The largest peak of radioactivity cochromatographed with authentic diacylglyceryl-AEP. A peak of radioactivity cochromatographed with the lyso derivative, monoacylglyceryl-AEP, and the radioactivity near the origin cochromatographed with free AEP in both this chromatographic solvent and solvent I. The radioactivity at the origin also remained at the origin when run in solvent I, and this compound has not been identified. It is interesting to note that here, as in our previous

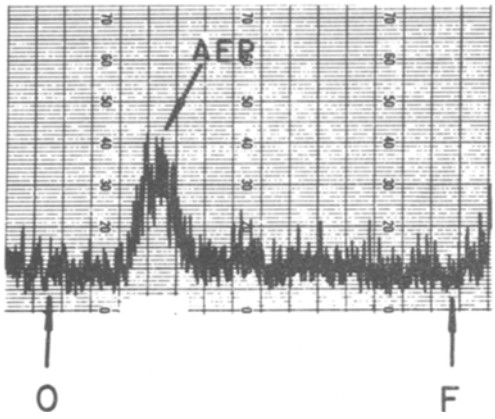

FIG. 5. Radiochromatogram of acid hydrolysate of liver lipids of a rat sacrificed 30 hr after injection with [G-^{3}H] aminoethylphosphonic acid (9.4 x 10^7 dpm; specific activity: 5.0 x 10^8 dpm/μmole). The H_2O soluble products of the acid hydrolysis of rat liver lipids were chromatographed in solvent I, isopropanol/acetone/58% NH_4OH/H_2O (5:2:4:3 v/v) (17) vs. authentic AEP. O and F indicate the origin and solvent front, respectively. Similar radiochromatograms were obtained at all time points studied.

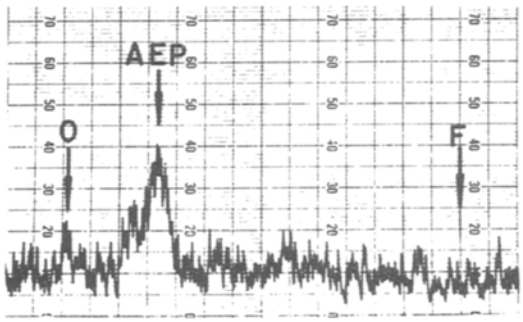

FIG. 6. Radiochromatogram of urine of rat injected with [G-^{3}H] aminoethylphosphonic acid (9.4 x 10^7 dpm; specific activity: 5.0 x 10^8 dpm/μmole). Urine samples of a 30 hr metabolic study were chromatographed in solvent I, isopropanol/acetone/58% NH_4OH/H_2O (5:2:4:3 v/v) (17) vs. authentic AEP. O and F indicate the origin and solvent front, respectively.

studies (13), no radioactivity was detected which cochromatographed with lecithin.

Hydrolysis experiments were performed on liver lipid samples of [G-^{3}H] AEP-injected rats in order to verify the identification of the radioactive lipids. After phospholipase A_2 (32) or phospholipase D (33) treatment, the lipids were chromatographed vs. standards in solvent II, and the results of the radiochromatogram scannings are shown in Figure 4. After phospholipase A_2 treatment, the radioactivity co-

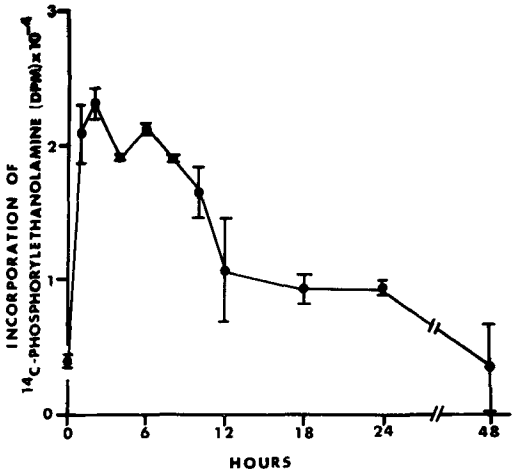

FIG. 7. Extent of incorporation of [$^{14}C_{1,2}$] phosphorylethanolamine into rat liver lipids. (8.3 x 10^5 dpm injected; specific activity: 206 dpm/nmole). Two rats were used for each time point. The values plotted are the dpm/g liver vs. time after injection.

chromatographing with diacylglyceryl-AEP decreased, while the radioactivity cochromatographing with the lyso derivative increased (Fig. 4B). No change was seen in the radiochromatogram scanning after phospholipase D treatment, which was consistent with the presence of a direct carbon to phosphorus bond (Fig. 4C). Samples of the liver lipids were hydrolyzed with 6N HCl for 8 hr in an autoclave (20), and the watersoluble fraction chromatographed vs. authentic AEP in solvent I. As shown in the strip scanning in Figure 5, the radioactivity cochromatographed with the AEP standard.

Two rats were injected intraperitoneally with [G-^{3}H] AEP (9.4 x 10^7 dpm) and maintained in metabolic cages for 30 hr so that urinary and fecal excretion of the ^{3}H-AEP could be determined. An average of 34% of the injected radioactivity was recovered in the urine. A sample of the urine was chromatographed in solvent I, and most of the radioactive material was found to cochromatograph with authentic AEP (Fig. 6). Approximately 10% of the injected radioactivity was recovered in the feces. The recovery of radioactivity in the urine is greater than that previously reported by our laboratory from studies using intravenous injection (13) and agrees with the results of others (14).

^{14}C-Phosphorylethanolamine Time Course Studies

Twenty-two rats, previously starved for 12-18 hr, were injected intraperitoneally with [$^{14}C_{1,2}$] phosphorylethanolamine (8.3 x 10^5

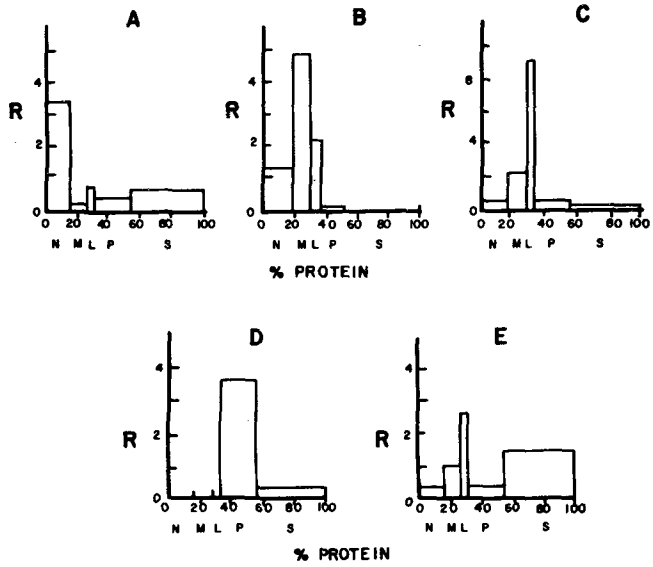

FIG. 8. Distribution pattern of subcellular markers. The individual graphs are A, DNA; B, cytochrome oxidase; C, N-acetyl-glucosaminidase; D, NADPH cytochrome c reductase; and E, catalase. The ordinant, R, is the total relative activity of each fraction divided by the total relative protein of that fraction. The abscissa is the percent protein in each fraction. N is the nuclear fraction; M, the mitochondrial; L, the lysosomal, P, the polysomal; and S, the soluble fraction.

dpm; specific activity: 1.98 x 10^{-5} dpm/μmole). At each of 11 designated time points within a 48 hr period, two injected rats were sacrificed, the livers removed and rinsed, lipids extracted, and the radioactivity of the fractions determined as described in Materials and Methods. The maximum incorporation of ^{14}C-phosphorylethanolamine into liver lipids occurred by 2 hr, at which time ca. 22% of the total injected radioactivity was recovered in the whole livers (Fig. 7).

Subcellular Fractionation

Subcellular fractionation was performed on the livers of rats, each of which had received [G-^{3}H] AEP (9.4 x 10^7 dpm; specific activity: 5.0 x 10^8 dpm/μmole), 12, 30, 36 or 48 hr prior to sacrifice. Various marker enzymes were assayed in the subcellular fractions. The distribution patterns of the subcellular markers are shown in Figure 8. DNA was a marker for the nuclear fraction (N), cytochrome oxidase for the mitochondrial fraction (M), N-acetylglucosaminidase for the lysosomal fraction (L), and NADPH cytochrome c reductase for the microsomal or polysomal fraction (P). Catalase was recovered for the most part in the soluble fraction (S), with the next highest amount being found in the lysosomal fraction (L).

Lipids were extracted from the subcellular fractions by the single phase procedure of Bligh and Dyer (30). Aliquots of the lipid fractions, and also of the unextracted fractions, were assayed for radioactivity, and the results are shown in Figure 9. The majority of the radioactivity in the unextracted subcellular fractions was recovered in the soluble fractions (S). However, most of the radioactivity present in the lipids was found in the polysomal fractions (P), with the next highest recovery in the nuclear (N) and mitochondrial (M) fractions.

DISCUSSION

At the 24 to 30 hr time point of the investigation, an average of 30-35% of the radioactive aminoethylphosphonic acid (AEP) was recovered in the whole blood and tissues studied. A 30 hr metabolic experiment demonstrated that an average of 45% of the injected radioactivity had been excreted in the urine and feces up to that point. Thus, an 80% recovery of the injected labeled AEP was obtained at the 24 to 30 hr time point. It is possible that the remaining 20% of the injected radioactive compound was localized in the skin, bone or intestine (tissues which were not studied), tissues in which newly synthesized phospholipids have been localized (34-36).

When [G-^{3}H] AEP was injected into rats and the liver lipids isolated, it was found that the largest amount of radioactivity was incorporated into diacylglyceryl-AEP, the phosphonate

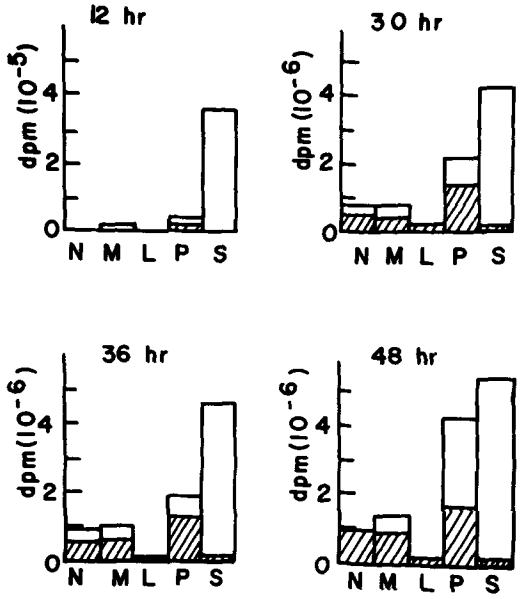

FIG. 9. [G-^{3}H] aminoethylphosphonic acid incorporation into subcellular fractions. Subcellular fractionation was performed on the livers of rats, who had received [G-^{3}H] AEP (9.4 x 10^7 dpm; specific activity; 5.0 x 10^8 dpm/μmole), 12, 30, 36 or 48 hr prior to sacrifice. The radioactivity of the subcellular fractions was determined before lipid extraction (open bars). In addition, the radioactivity of the lipid in each fraction was also determined (cross-hatched bars). N is the nuclear fraction; M, the mitochondrial; L, the lysosomal; P, the polysomal; and S, the soluble fraction. The time after injection with ^{3}H-AEP is indicated.

analogue of phosphatidylethanolamine. Some AEP was also incorporated into the lyso analogue and an unidentified compound. In all cases, no other radioactive lipids could be detected, including the phosphonate analogue of phosphatidylcholine. The lack of any methylation of diacylglcyeryl-AEP was also found to be the case in studies with *T. pyriformis* (20,37). When N-trimethyl-2-aminoethylphosphonic acid (cholinephosphonic acid) was injected into rats and incorporated into the phospholipid fraction, no demethylation of the phosphonolecithin was seen (15). Thus, it appears that an alkylphosphonic acid, AEP or N-trimethyl-AEP, can be incorporated into the liver lipids of a rat, but that no enzyme system exists for the methylation or demethylation of these phosphonolipids. It was also noted that at no time in the investigation was there evidence of any cleavage of the carbon to phosphorus bond, since the only radioactive compounds detected were derivatives of AEP.

In contrast to the results with radioactive AEP, the incorporation of ^{14}C-phosphorylethanolamine into liver lipids reached a maximum only 2-3 hr after administration. It is possible that the enzymes involved in phosphonolipid metabolism have slower rates of reaction than the corresponding enzymes of phospholipid metabolism. A more likely possibility is that the same enzyme system is involved in both phospholipid and phosphonolipid metabolism, but that AEP is incorporated in a less efficient manner than are the precursors of phospholipids.

Three months after the injection of ^{3}H-AEP, little radioactivity remained in the liver lipids or lipid-free residues. The radioactivity present in the whole blood, however, remained essentially the same throughout the three month time period. The length of time the radioactivity remained in each fraction could be a function of the turnover time of that fraction. The half-life of diacylglyceryl-AEP has been reported to be between 100 and 140 hr (38). The half-life of the proteins of the liver has been reported as 90 to 95 hr (39). These half-life values would account for the fact that little radioactivity remained in the lipid or lipid-free residues after 2 wk. On the other hand, the turnover time of the rat erythrocyte has been found to be ca. 100 days (40). After 3 months, therefore, one might expect a substantial amount of the original radioactivity to still be present in the whole blood samples, as was found in these studies.

In the subcellular fractionation studies, the distribution of subcellular markers was similar to that reported by other researchers (24-27) (Fig. 8). The cytochrome oxidase data showed that there was some mitochondrial (M) contamination of the nuclear (N) and lysosomal (L) fractions. The total radioactivity of the subcellular fractions at the various time points was determined, and most of the radioactivity was found in the soluble fraction (Figure 9).

At the 12 hr time point, little ^{3}H-AEP was detected in any other subcellular fraction, while at the 30, 36, and 48 hr time points, increasing amounts of radioactivity were found in other fractions, particularly the nuclear, mitochondrial, and microsomal or polysomal (P) fractions. The fact that the first radioactivity detected was presented in the soluble fraction could indicate that the ^{3}H-AEP present in the soluble fraction was a precursor of the radioactive material of the other subcellular fractions.

When lipids were extracted from the various subcellular fractions of the 12, 30, 36, and 48 hr rats, very little labeled lipid material was found at the 12 hr time point (Fig. 9). All of

the ^{3}H-AEP lipid present was found in the polysomal fraction. At the 30, 36, and 48 hr time points, a large amount of radioactivity was found to be incorporated into lipid. Most of the radioactivity was associated with the polysomal fraction, with the next highest amounts in the nuclear and mitochondrial fractions. Since the ^{3}H-AEP lipid was first seen in the polysomal fraction, it is possible that the synthesis of phosphonolipid took place in this fraction. The polysomal or microsomal fraction has been found to be the predominant site of phospholipid synthesis (41,42). The ^{3}H-AEP lipid found in the mitochondrial fraction was most likely due to an exchange of phosphonolipid between the polysomes and the mitochondria, while the radioactive lipid of the nuclear fraction was probably for the most part the result of mitochondrial contamination. The exchange of phosphonolipid between the microsomes and mitochondria has been demonstrated by other researchers (38) and the exchange of phospholipid has also been reported by many laboratories (41, 43-45). The pattern of radioactivity found in the present investigations could also indicate the localization of phosphonolipids in the plasma membrane, since plasma membrane fragments are found, for the most part, in the nuclear and polysomal fractions (46).

Phosphorylethanolamine is incorporated into phosphatidylethanolamine by a pathway involving a CDP intermediate (12). The results of other suggest that alkylphosphonic acids may be incorporated into lipids by a similar mechanism (11,15,16) involving the enzyme CTP-cytidyltransferase and the reaction:

$$\text{AEP} + \text{CTP} \rightarrow \text{CMP-AEP} + \text{PP}_i$$

It has been reported that the CTP-cytidyltransferase of phospholipid metabolism is primarily located in the soluble fraction of the liver (47). It appears that the phosphotransferase, which would catalyze the reaction:

$$\text{CPM-AEP} + \text{Diglyceride} \rightarrow \text{Diacylglyceryl-AEP} + \text{CMP}$$

could be located in the polysomal fraction, since it is in this fraction that the presence of phosphonolipid is first detected. The microsomal (or polysomal) fraction has been shown to be the site of synthesis of many phospholipids (45).

ACKNOWLEDGMENTS

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Serum Lipoproteins as Inhibitors of Haemagglutination by Rubella Virus

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ABSTRACT

The role of serum lipoproteins as non-antibody-like inhibitors of haemagglutination for rubella virus was investigated. Dissociation of lipoproteins into their respective lipid and protein components significantly reduced their inhibitory titre. This reduction was more prominent with the protein component. When mixtures of pure lipids were tested for their haemagglutination inhibitory activities, no inhibition was observed. Sonication of the three major lipoprotein fractions significantly increased the inhibitory activities of low density lipoproteins (LDL) and very low density lipoproteins (VLDL) but not high density lipoproteins (HDL). Succinylation, acetylation, and methylation of the lipoproteins did not appear to affect their inhibitory titre. On the other hand, phospholipase A treatment significantly increased the inhibitory properties of all lipoproteins fractions. These results are discussed in terms of the possible mechanisms by which the lipoproteins interact with the rubella haemagglutinins.

INTRODUCTION

Information on the nature of lipoproteins and lipid mixtures acting as non-antibody inhibitors of haemagglutination of certain categories of lipid-containing viruses is inconsistent and not well established. Gorman (1) and more recently Halonen et al. (2) have shown that pure lipid mixtures containing either phospholipid alone or a combination of phospholipid and cholesterol had the highest inhibitory activity for a number of arboviruses and rhabdoviruses. Since, in the latter study, native human serum lipoproteins have also been demonstrated to inhibit the haemagglutination of these viruses, the authors suggested that the lipid moiety of these macromolecular complexes is probably responsible for interaction with the viral haemagglutinin.

In a previous study, we systemically examined the major human serum lipoprotein fractions for their haemagglutination inhibitory activity for two different togaviruses, viz., Japanese encephalitis and rubella viruses (3). Both of them were found to be sensitive to low density lipoprotein (LDL, d 1.006-1.063 g/ml) and very low density lipoprotein (VLDL, d<1.006 g/ml) but only weakly so to high density lipoprotein (HDL, d 1.063-1.21 g/ml). Since HDL has the highest content of phospholipid and cholesterol per unit lipoprotein weight (4), the inability of this lipoprotein fraction to inhibit haemagglutination appears to be at variance with results obtained when purified lipid fractions were used. Nevertheless, because there are basic differences in the structure of human serum lipoproteins and artificially prepared lipid mixtures, it would be premature to draw any conclusions on the nature of the binding group without first examining the role of lipoprotein structure in the interaction with the virus haemagglutinin.

In the present study, we have extended our investigation to identify the roles of lipid and/or protein components of the three major lipoprotein fractions in inhibiting the haemagglutination of rubella virus. Similar studies were also carried out with Japanese encephalitis virus and their results will be reported elsewhere (5).

MATERIALS AND METHODS

Chemicals

All chemicals used were reagent grade. Purified phospholipase A (EC 3.1.1.4), C (EC 3.1.4.3), and D (EC 3.1.4.4) as well as chromatographically pure cholesterol, cholesteryl oleate, triolein and egg lecithin were obtained from Sigma Chemical Co., St. Louis, MO, and were used without any further purification. Precoat thin layer chromatographic strips (Silica Gel G) were from A.H. Thomas Co., Philadelphia, PA. Precast agarose slides for lipoprotein electrophoresis were from Bio-Rad Labs, Richmond, CA.

Preparation of Serum Lipoproteins and Apolipoproteins

Pooled serum was prepared from blood drawn from three normal healthy male and female donors after fasting for at least 12 hr. The different lipoprotein fractions, i.e., the VLDL, LDL, and HDL, were isolated from the

freshly prepared sera by sequential ultracentrifugation at the density intervals of d<1.006 g/ml, d 1.006-1.063 g/ml, and d 1.063-121 g/ml, respectively, as described previously (3).

Lipoprotein fractions were delipidated by organic solvent extraction following essentially the method of Shore and Shore (6). The lipoprotein-proteins obtained were not completely soluble in Tris saline buffer (0.01 M Tris HCl buffer, pH 7.4, containing 0.19 M NaCl and 0.1 mg/ml EDTA) and were solubized in 0.1 M Tris HCl buffer, pH 8.2, containing 0.002 M SDS (7). The apolipoprotein fractions prepared are designated apoHDL apoLDL and apoVLDL according to the source of the original material.

Preparation of Lipid Extracts and Dispersions

Lipids of the different lipoprotein fractions were extracted in a mixture of chloroform and methanol 1:1 v/v by a modified procedure of Sperry and Brand (8). Lipid dispersions were prepared with (a) the extracted lipoprotein lipids, (b) mixtures of commercial lipids, in concentrations and molar ratios as indicated in the tables, by pipetting a known amount of lipid dissolved in chloroform into a conical sonication vial. The organic solvent was evaporated under a gentle stream of N_2. An exact volume of Tris saline buffer was added to obtain the appropriate final concentration. The mixture was sonicated in an ice bath for a total of five 1-min pulses with 1-min intervals using a Branson Sonifier (Heat Systems Co., Melville, Long Island, NY). The lipid dispersions prepared by this method were usually used within one day for determining inhibitory activity. Prolonged storage of some of the lipid dispersions, particularly those containing apolar lipids, produced inconsistent results. No evidence of denaturation was obtained when the sonicated lipids were analyzed by thin layer chromatography.

Perturbation of Lipoprotein Structure

Sonication: Five ml aliquots of serum lipoprotein solutions, containing 1 mg protein per ml, in Tris saline buffer were sonicated as described above. A trace amount of denatured material was generated during sonication and was removed by low speed centrifugation. Recovery of the protein and lipid in the supernatant was usually greater than 95%. Double gel immunodiffusion of the sonicated LDL and VLDL against anti-β-lipoprotein serum yielded nonidentical precipitin lines with the native lipoproteins.

Chemical modification: Methylation, acetylation, and succinylation of the serum lipoprotein fractions were performed essentially as described by Means and Feeney (9), Riodan and Vallee (10) and Scanu (11), respectively. The degree of modification was assessed by electrophoresis in agarose (12). In the case of succinylation and acetylation, there was a significant increase of electrophoretic mobility towards the anode. Immunodiffusion against anti-β-lipoprotein serum was carried out on the succinylated and acetylated LDL and VLDL and the precipitin lines produced by these fractions did not fuse with those produced by their native materials.

Phospholipase treatment: Purified lipoprotein fractions, containing 1 mg protein per ml, in 0.05 M Tris HCl buffer, pH 7.4, containing 0.005 M $CaCl_2$ were incubated with either phospholipase A, C, or D at 37 C overnight in an enzyme concentration of 0.05 mg/ml. Aliquots of the enzyme treated material were taken for inhibitory activity determination, lipid analysis, electrophoresis, and immunodiffusion assay. In control experiment, samples containing phospholipase did not have a deleterious effect on the performance of the haemagglutination inhibition test.

Methods of Analysis

Protein and total lipid determinations were performed as described previously (3). Lipid phosphorous was determined by the method of Dryer et al. (13). Lipid components from the total lipid extracts were separated by thin layer chromatography on Silica Gel G strips using a solvent system of hexane/ether/acetic acid 60/40/1 v/v (14). Double immunodiffusion assay was performed essentially as described by Hatch and Lees (15) in 1% agarose plates.

Haemagglutination Inhibition Test

These were performed by established protocol (16) using "V" microplates (Cooke Microtiter System, Dynatech Laboratories, Alexandria, VA). Test samples of inhibitor for assay were initially diluted as required in the first well of the plate and then submitted to doubling dilutions in 25 μl volumes in HSAG buffer (pH 6.2, 0.6% HEPES, 0.8% NaCl, 0.01% $CaCl_2 \cdot 2H_2O$, 1% bovine albumin Fraction V, 0.003% gelatin) using a Cooke Microtiter System. To each dilution was added 25 μl rubella antigen preparation (Baylor strain; from Grand Island Biological Co., Oakland, CA) previously standardized by titration with erythrocytes to contain 4 units of haemagglutinating activity per 25 μl. The plates were shaken to facilitate mixing and then held at room temperature (ca. 25 C) for 15 min to allow antigen and inhibitor to combine; the

TABLE I

Comparison of the Activities of Serum Lipoprotein Fractions and Their Lipid and Protein Components in the Inhibition of Haemagglutination by Rubella Virus

Lipoprotein[a] fraction	Protein conc. (mg/ml)	Lipid conc. (mg/ml)	Inhibitory activity
HDL	1	1.23	<5
apoHDL[b]	1	--	10
HDL-lipid	-	1.47	64
LDL	1	4.85	160
apoLDL[b]	1	--	15
LDL-lipid	-	4.70	128
VLDL	1	7.31	160
apoVLDL[b]	1	--	10
VLDL-lipid	-	6.75	128

[a]HDL = high density lipoprotein; LDL = low density lipoprotein; VLDL = very low density lipoprotein.

[b]These apolipoproteins were dissolved in 0.1 M Tris HCl buffer, pH 8.2, containing 0.002 M SDS. Although the buffer alone did not produce a titre >5, the possibility that an SDS-apolipoprotein complex was formed when the apolipoproteins were dissolved in this buffer was not excluded. Thus, the true inhibitory activities of these apolipoprotein fractions may not be identical to the values presented.

TABLE II

Haemagglutination Inhibitory Activity of Sonicated Dispersions of Mixtures of Purified Lipids

Lipid mixture[a]	Appearance	Inhibitory activity
Lecithin	Opalescent	<5
Lecithin/triolein	Turbid	<5
Lecithin/cholesteryl oleate	Turbid	<5
Lecithin/cholesterol	Opalescent	<5

[a]The lipid concentration in all the samples was 2 mg/ml. The molar ratio of the lipids was 1:1.

smaller inhibitor molecules effectively coat the surface of the virus haemagglutinating antigen to block or inhibit its union with erythrocytes. To each well was added 50 µl trypsinized human group O erythrocytes suspended in 0.2 M phosphate buffer, pH 6.0 (17) for agglutination by free uncoated virus antigen and the trays held at 4 C for 2 hr. The inhibitory titre of the sample under test was the reciprocal of the highest dilution of inhibitor which caused 50% inhibition of haemagglutination. The 50% endpoint was obtained by interpolation and was the dilution halfway between that showing no haemagglutination and the one with complete haemagglutination. Positive and negative controls were included for each tray to facilitate visual interpretation of the patterns. All titrations were performed in duplicate and in each case yielded identical results. Because of the subjective nature of interpretation of these haemagglutination patterns, only fourfold differences in inhibitory titre corresponding to doubling dilutions in two wells, or greater, are generally considered to indicate significant differences from one sample to another.

RESULTS

The results of an experiment designed to evaluate the role of lipid and protein in promoting the inhibition of haemagglutination by rubella virus are summarized in Table I. When the three major lipoprotein fractions were delipidated by organic solvent extraction and their respective lipid and protein moieties tested for inhibition of haemagglutination, no significant change of activity was observed with LDL- and VLDL-lipids. On the other hand, a ten-time reduction of activity was observed for apoLDL and apoVLDL. In contrast, HDL-lipid showed a significant rise in inhibitory activity compared with native HDL.

Since the lipoprotein-lipids alone expressed appreciable inhibitory activity, we examined the possible role of different lipid components in the serum lipoproteins as inhibitors of haemagglutination. Sonicated dispersions of purified lipid mixtures containing the different

TABLE III

Effect of Sonication on Serum Lipoprotein Haemagglutination Inhibitory Activity

Lipoprotein class[a]	Appearance	Inhibitory activity
HDL	Clear	<5
Sonicated HDL	Clear	<5
LDL	Clear	160
Sonicated LDL	Turbid	480
VLDL	Opalescent	160
Sonicated VLDL	Very turbid	640

[a]All lipoprotein fractions assayed were 1 mg/ml with respect to protein. HDL = high density lipoprotein; LDL = low density lipoprotein; VLDL = very low density lipoprotein.

TABLE IV

Effect of Chemical Modification on Serum Lipoprotein Haemagglutination Inhibitory Activity

Lipoprotein class[a]	Inhibitory activity	Immunodiffusion characteristics[b]	Relative electrophoretic mobility
HDL	<5	nt	1.00
Methylated HDL	<5	nt	1.04
Acetylated HDL	<5	nt	1.65
Succinylated HDL	<5	nt	1.62
LDL	80	same	1.00
Methylated LDL	40	same	1.16
Acetylated LDL	80	altered	2.75
Succinylated LDL	80	altered	3.00
VLDL	160	same	1.00
Methylated VLDL	120	same	1.06
Acetylated VLDL	240	altered	1.60
Succinylated VLDL	240	altered	2.17
Albumin	<5	nt	nt
Methylated albumin	<5	nt	nt
Acetylated albumin	<5	nt	nt
Succinylated albumin	<5	nt	nt

[a]The protein concentration in all samples assayed was 1 mg/ml. The lipid concentrations for HDL, LDL, and VLDL were ca. 1, 3, and 7 mg/ml. HDL = high density lipoprotein; LDL = low density lipoprotein; VLDL = very low density lipoprotein.

[b]Same indicates that the precipitin line formed by the sample fused with the native material when assayed by the immunodiffusion technique as described in Materials and Methods; nt, not tested.

major lipoprotein lipids with lecithin as a stabilizer (molar ratio 1:1) were tested for their abilities to inhibit haemagglutination. A inhibitory titre of <5 observed in all cases (Table II). Based on these results, it is apparent that the specificity of the haemagglutination inhibition process cannot be depended entirely on any one of the major lipid components of the serum lipoprotein.

In the previous experiment when the lipid and protein components of LDL and VLDL were separated and tested individually for their haemagglutination inhibitory activity, a loss of activity was observed relative to that of the native material. In preliminary experiments, attempts to restore the original inhibitory activity by recombining the separated lipid and protein components proved unsuccessful. The experimental conditions used to effect native configuration may not have been appropriate or else the separated components may have been altered structurally so as to render this impossible. Since lipoprotein structure may play a role in the inhibition of haemagglutination by rubella virus, we attempted to use milder procedures to alter the structure of the lipoproteins with a view to assess something of the nature of the interaction between the virus haemagglutinin and the lipoproteins.

When serum lipoproteins are subjected to ultrasonic irradiation, removal of lipid and/or protein from the lipoprotein surface may results (18). Because of the potential effect of this method in exposing or affecting receptor groups on the lipoprotein surfaces, we sonicated the three major lipoprotein fractions and

tested their inhibitory activity against rubella virus. No alteration of HDL inhibitory activity was observed before and after sonication (Table III). On the other hand, sonication of LDL and VLDL significantly increased their inhibitory titre, VLDL being the more prominent of the two.

To assess the role of surface charge in the interaction between lipoproteins and haemagglutinin, the three major lipoprotein fractions were chemically modified as shown in Table IV. Modifications of HDL, LDL, VLDL, and human serum albumin (included as a background control) had little effect in altering the inhibitory properties of these fractions.

Phospholipids are well recognized as one of the most crucial structural components of serum lipoproteins (19). To understand further the role that phospholipids play in the interaction between lipoprotein and the virus haemagglutinin, we treated the purified lipoprotein fractions with different phospholipases and observed changes (Table V) associated with such treatments. With phospholipase A, 96-100% of the phospholipids associate with the three major lipoprotein fractions reacted. Significant increases in inhibitory activity were observed in all fractions. By contrast, although a significant proportion of phospholipid in LDL and VLDL reacted on phospholipase C treatment, no significant change in inhibitory activity associated with these fractions was apparent. When HDL, LDL, and VLDL were treated with phospholipase D, only 0, 22, and 11%, respectively, of the phospholipids in these lipoprotein fractions reacted. As in the previous case, no general trend in altered inhibitory activity could be established.

TABLE V

Effect of Phospholipase Treatment on Serum Lipoprotein Haemagglutination Inhibitory Activity

Lipoprotein class[a]	Type of treatment	Inhibitory activity	Immunodiffusion characteristics[b]	% substrate reacted	Relative electrophoretic mobility
HDL	none	<5	nt	nt	1.00
	Phospholipase A	60	nt	100	1.39
	Phospholipase C	<5	nt	20	1.23
	Phospholipase D	<5	nt	0	1.15
LDL	none	80	same	nt	1.00
	Phospholipase A	320	same	100	2.45
	Phospholipase C	30	same	88	1.45
	Phospholipase D	80	same	22	1.09
VLDL	none	80	same	nt	1.00
	Phospholipase A	1300	same	96	2.06
	Phospholipase C	40	same	52	1.00
	Phospholipase D	40	same	11	1.12

[a]Protein concentration in all the samples assayed was 1 mg/ml. The lipid concentrations for HDL, LDL, and VLDL were ca. 1, 3, and 7 mg/ml. HDL = high density lipoprotein; LDL = low density lipoprotein; VLDL = very low density lipoprotein.
[b]See footnote of Table IV.

DISCUSSION

This work confirms that lipoproteins mediate non-antibody inhibition of haemagglutination by rubella virus. Upon dissociation of the lipid and protein components, reduction of activity, particularly with the apolipoproteins, resulted. In contrast to the studies carried out by Gorman (1) and Halonen et al. (2) on arboviruses and rhabdoviruses, our data do not support the idea that different lipid components of the lipoproteins could solely account for the total inhibitory activity produced by the native material. Nevertheless, since the viruses used in their studies were not the same as ours, it is uncertain whether this discrepancy is due to basic differences in the interaction of the viruses with the lipoproteins and their constituents or to variations of experimental procedures, particularly in the prepara-

tion of lipid dispersions.

Based on the data presented in Table I, lipids apparently play a role in mediating the inhibition of haemagglutination by rubella virus. However, the inability of pure lipid dispersions to inhibit haemagglutination (Table II) suggests that the mechanism of inhibition might require factors other than any one of the major lipids alone. This contention is at least partially supported by the fact that the inhibitory activity of lipoproteins was increased after sonication. The mechanism by which sonication promoted the inhibitory activities of LDL and VLDL is uncertain. Nevertheless, this treatment has apparently changed the immunodiffusion characteristics of these lipoproteins and suggests that the lipoprotein structure has been altered, which may be crucial in exposing potential sites for interaction. In support of this idea, sonication of serum lipoproteins has been shown to alter the surfaces of these macromolecules. Physical and chemical analysis of the sonicated products indicate that there is probably a reorganization of lipoprotein structure with the subsequent removal of lipids from the lipoproteins (18). Unfortunately, the results of the present study could not provide an unequivocal answer on the origin of the increase of inhibitory activity in the final sonicated products. Shortridge et al. (20) proposed that in the case of LDL a galactosamine moiety might play a role for the inhibition of rubella virus haemagglutination. If this is the case, sonication might bring about a favorable conformational change so as to allow a better interaction between the virus haemagglutinin and the lipoprotein.

When the different lipoprotein fractions were acetylated or succinylated, there was an apparent increase of net negative charge on these macromolecules. Since there was no reduction of inhibitory activity in these cases, it would seem unlikely that an ionic type of interaction involving charged groups was crucial for the binding of the viral haemagglutinin to the lipoproteins.

When the lipoproteins were treated with phospholipases A, C, and D, only phospholipase A significantly increased the inhibitory titre. The by-products of the reaction catalyzed by this enzyme are lysolecithin and free fatty acid. Since both of these compounds have relatively strong surface activity, it is possible that they might induce alteration of both protein conformation and lipid packing in the lipoproteins. Verdery and Nichols (21) have recently shown that lysolecithin has the ability to induce conformational changes when combined with apolipoproteins. Hence, it is possible that the lysolecithin generated by phospholipase A treatment may somehow be connected with the exposure of previously hidden binding sites.

Although native HDL was devoid of detectable inhibitory activity, significant increases in activity were observed when the extracted lipid components were tested. Moreover, similar increases in activity were observed when this lipoprotein fraction was subjected to phospholipase A treatment. These observations clearly indicated that the native HDL contains the potential sites for interacting with the virus haemagglutinin. However, their accessibility or exposure could be achieved only after the native lipoprotein was modified. Further studies in the origin of inhibitory activity in the HDL fraction will probably yield important information on the nature of the binding group for the virus haemagglutinin.

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Preparation of Specifically Dideuterated Octadecanoates and Oxooctadecanoates[1,2]

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ABSTRACT

Sixteen methyl *gem* dideuteroctadecanoates with two deuterium atoms at positions 2- to 17- and seven oxo esters, 8-oxooctadecanoate-5,5-d_2, 8-oxooctadecanoate-11,11-d_2, 11-oxooctadecanoate-8,8-d_2, 11-oxooctadecanoate-14,14-d_2, 12-oxooctadecanoate-9,9-d_2, 7-oxooctadecanoate-10,10-d_2 and 13-oxooctadecanoate-16,16-d_2 with two deuteriums on the carbon γ to the oxo group, have been synthesized. Two principal methods of introducing deuterium were used: preparation of 2,2-dideutero acids by exchange with deuterium oxide followed by chain extension giving dideuteroxooctadecanoates, which were then reduced, as tosylhydrazones, with sodium cyanoborohydride to dideuterooctadecanoates and stepwise introduction by reduction of oxooctadecanoates with sodium borodeuteride, formation of tosylate or mesylate, reduction with lithium aluminum deuteride to tetradeuterooctadecanol and oxidation to dideuterooctadecanoic acid.

INTRODUCTION

Deuterium labeled fatty acids which are mono- or, preferably dideuterated, at specific carbons are extremely useful in investigations of ^{13}C NMR spectra of lipids. Besides affecting the signal of the deuterated carbon, the signals of the α and β carbons are also changed in such a way that they can be recognized (1,2). Studies of lipid membrane structure by ^{2}H NMR have also been made using deuterated fatty acids (3,4).

The *gem* dideuterooctadecanoic acids, octadecanoic-3,3-d_2 to -17,17-d_2 acids, were previously prepared by Dinh-Nguyen (5) by anodic coupling of 2,2-dideuterated acids with dicarboxylic acid half methyl esters. However, since 2,2-dideuterated acids could not be prepared with high isotopic purity at that time, the products were incompletely labeled; also the yields were relatively low. The present paper describes and compares preparations of the sixteen isomeric dideuterooctadecanoates in high isotopic purity using a number of different methods. Seven specifically dideuterated oxooctadecanoates with both deuteriums attached to the carbon γ to the oxo group have also been synthesized. These products and the derived hydroxy and acetoxy esters can be employed to assign signals in ^{13}C NMR spectra of oxygenated C_{18} esters.

EXPERIMENTAL PROCEDURES

Analysis of Product

^{1}H NMR spectra were measured in carbon tetrachloride solution with a Varian HA-100 spectrometer. Gas liquid chromatography (GLC) was performed using a column packed with 2% silicone SE-30 on 80-100 mesh Gas Chrom Q, and the temperature was programmed from 125 to 325 C. Mass spectra were measured with an MS-12 mass spectrometer at the Department of Chemistry, University of Saskatchewan.

Starting Materials

C_4 to C_{16} saturated acids or esters were commercial products which, according to GLC, did not contain more than 2% of other chain lengths. Octadecanoic acid was prepared by hydrogenation of "Pamolyn" oleic acid (Hercules Inc., Wilmington, DE). Oleic acid was prepared by purification of commercial acid to obtain a monoenoic acid which, however, according to oxidative cleavage, contained 4% of 11-octadecenoic acid. Petroselinic acid ("95%", Sigma Chemical Co., St. Louis, MO) was purified (65% recovery) by crystallization from 90% ethanol (6); ^{13}C NMR indicated that positional isomers were absent. Sodium 10-undecenoate (Sigma Chemical Company) was used without purification. Methyl 6- and 7-oxooctadecanoates were prepared by enamine synthesis with 1-morpholinocyclopentene (7) and 1-morpholinocyclohexene (8), respectively, and methyl 13-oxohexadecanoate and 13-oxooctadecanoate were obtained by enamine synthesis using 1-morpholinocyclododecene (9). Methyl 12-oxooctadecanoate was obtained by chromic acid oxidation of hydrogenated

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[2]NRCC No. 15663.

castor oil esters and purified by column chromatography. Methyl 17-oxooctadecanoate was prepared by oxidation of 17-hydroxyoctadecanoate obtained by fermentation of methyl stearate (10).

Preparation of 2,2-dideutero Acids

Acids (0.1 mole) were neutralized with 0.1 M NaOH in sufficient H_2O to give a clear solution at 90 C (10 ml for C_4 acid, 150 ml for C_{16} acid), and H_2O was removed by freeze drying to constant weight. A mixture of sodium salt and D_2O (100 ml) containing NaOD (1 g) was then heated in a rocking stainless steel autoclave at 200 C for 3 days. Methyl esters were also used in place of sodium salts with sufficient NaOD in D_2O (100 ml) to give a solution of the salt in 1% NaOD. When larger quantities were required, 0.25 moles of sodium salt were used, D_2O was removed by freeze drying after one exchange and the reaction repeated with fresh D_2O (100 ml). The product was acidified with concentrated HCl and acids extracted with chloroform; the extract was washed 3 times with H_2O to ensure complete conversion of the COOD group to COOH. Acid was distilled and a portion converted to methyl esters, for MS analysis, with CH_2N_2 in CH_2Cl_2.

Reduction with $NaBD_4$

Methyl oxo ester (0.1 mole) was stirred in MeOH (300 ml) at 20 C when partial solution occurred; $NaBD_4$ (0.063 mole) was added in portions during 15 min and stirring was continued for another 15 min. The solution became clear as oxo ester was converted to hydroxy ester. Excess reagent was decomposed with CH_3COOH, the reaction mixture poured into H_2O, and the deuterohydroxy ester extracted with $CHCl_3$. Yields were practically quantitative, and there was no evidence for exchange of protons on carbons α to the oxygenated carbon.

Preparation of *p*-toluenesulfonates

Solutions of deuterohydroxyoctadecanoate (0.1 mole) and tosyl chloride (0.5 mole), each in pyridine (300 ml), were cooled to 0 C, mixed and kept at 0 C for 2 days. The mixture was poured into ice water and tosylate extracted with ether; repeated washing with water removed most of the pyridine. After removal of the ether, tosylate was crystallized from hexane or acetone (for tosylate of methyl 17-hydroxyoctadecanoate-17-*d*). Melting points and yields were: methyl 7-hydroxyoctadecanoate-7-*d* *p*-toluenesulfonate, 35-36 C, 80%; methyl 12-hydroxyoctadecanoate-12-*d* *p*-toluenesulfonate, 38-39 C, 71%; methyl 17-hydroxyoctadecanoate-17-*d* *p*-toluenesulfonate, 68-69 C, 80%.

Preparation of Methanesulfonates (11)

Primary alkanol (0.1 mole) and triethylamine (0.15 moles) were dissolved in CH_2Cl_2 (600 ml), cooled to -10 C and a solution of methanesulfonyl chloride (0.14 mole) in CH_2Cl_2 (50 ml) was added with stirring. After keeping at 0 C for 2 days, the reaction mixture was washed 3 times with ice water (150 ml) and dried over sodium sulfate. C_4 to C_{11} primary alkyl mesylates were purified by distillation (12) and C_{16} and C_{18} mesylates by crystallization from methanol. Hexadecyl-1,1-d_2 mesylate had m.p. 52-53 C [literature (13) gives 54-55 C for undeuterated compound], *cis*-9-octadecenyl-2,2-d_2 methanesulfonate had m.p. ca. 5 C and *cis*-6-octadecenyl-2,2-d_2 methanesulfonate had m.p. 20-21 C. The yields were 77-100%.

Mesylates of deuterohydroxy esters were prepared in the same way. The melting points and yields were: methyl 13-hydroxyhexadecanoate-13-*d* methanesulfonate, 28-29 C, 87%; methyl 13-hydroxyoctadecanoate-13-*d* methanesulfonate, 18-20 C, 89%; methyl 6-hydroxyoctadecanoate-6-*d* methanesulfonate, 27-28 C, 80%.

Reduction with $LiAlH_4$ or $LiAlD_4$

Methyl esters: Methyl ester (0.1 mole) was refluxed 18 hr in ether (400 ml) with $LiAlH_4$ (0.075 moles); excess reagent was decomposed with H_2O and metal salts dissolved in 4 N H_2SO_4. Product was extracted with ether and did not require purification, yields were 90-100%.

Methyl ester tosylate or mesylate: Tosylate or mesylate (0.1 mole) was reduced in ether with $LiAlD_4$ (0.175 moles) as above. The yield of tetradeutero alcohol could not be estimated due to the presence of by-products.

Conversion of Tetradeutero Alcohols to Methyl Dideutero Esters

Tetradeutero alcohol obtained by reduction of 0.1 mole of tosylate or mesylate was dissolved in glacial acetic acid (1 liter) at 25 C, and a solution of chromium trioxide (0.16 moles) in water (20 ml) and acetic acid (200 ml) was added during 15 min with stirring at 25 C. After a further 15 min, the mixture was poured into 4 N H_2SO_4 (3 liters), excess oxidant was reduced with SO_2 and the product was extracted with $CHCl_3$. After CH_2N_2 treatment, GLC and thin layer chromatography (TLC) (hexane:ether 4:1) both showed the presence of C_{36} mono ester (when dideutero C_{18} acid was being prepared). The crude product was

refluxed with methanol containing 5% HCl for 18 hr giving a mixture of methyl dideutero ester, tetradeutero alcohol, and by-products. The mixture was chromatographed on Biosil A (200 g); elution with hexane-ether (99:1) gave methyl esters, elution with hexane-ether (98:2 to 95:5) gave by-products, and elution with hexane-ether (90:10 to 85:15) gave tetradeutero alcohol. The ratio of weight of ester to alcohol was about 3:1. Overall yields of dideutero esters from hydroxy esters were 33-48%, but when recovered alcohol was reoxidized and rechromatographed, it rose to 45-60%.

Reaction of Primary Alcohol Mesylates with Diethyl Malonate

The procedure of Spener and Mangold (14) was followed. When hexyl-2,2-d_2- and nonyl-2,2-d_2-malonic acids were prepared they were crystallized from benzene, yields were 83 and 57%, respectively. When acids containing two more carbons were prepared, crude malonic acid was heated at 160 C for 1.5 hr and the product distilled. The following acids were prepared (with yields from alcohol in parentheses): hexanoic-4,4-d_2 (67%); dodecanoic-4,4-d_2 (77%); 12-tridecenoic-4,4-d_2 (81%); octadecanoic-3,3-d_2 (85%); octadecanoic-15,15-d_2 (62%); *cis*-11-eicosenoic-4,4-d_2 (66%); *cis*-8-eicosenoic-4,4-d_2 (70%).

Preparation of Octadecanoic-4,4-d_2 Acid by Anodic Coupling

A solution of hexadecanoic-2,2-d_2 acid (0.05 mole) and methyl hydrogen succinate (0.1 mole) in 0.033 N methanolic sodium methoxide (80 ml) was electrolyzed between platinum electrodes for 16 hr at 1 amp. Further C_4 half ester (three 0.025 mole portions) was added during the course of the reaction. Solvent was removed and residue chromatographed on Biosil A (150 g). Elution with hexane gave hydrocarbons followed by crude product (5.93 g). Crystallization from methanol removed some shorter chain by-products and gave 4.22 g esters which were saponified. The soaps were extracted with hexane, the acid isolated, and crystallization from $CHCl_3$ gave octadecanoic-4,4-d_2 acid (2.8 g; 19.7%).

Preparation of Methyl Hydrogen Suberate

A suspension of suberic acid (0.575 moles) in toluene (350 ml) containing methanol (0.863 moles) and concentrated H_2SO_4 (1 ml) was stirred and heated at 90 C for 18 hr. The solution was cooled to 10 C, filtered from unreacted dibasic acid, and extracted twice with 3 N ammonium hydroxide (250 ml). The extract was acidified and crude half-ester extracted with warm hexane. After keeping at 20 C, the hexane extract was filtered and hexane removed; distillation gave half methyl ester (0.24 moles), b/6 mm 166-170 C. Methyl hydrogen undecanedioate was prepared in the same way.

Preparation of Acid Chlorides

Acid or half methyl ester (0.1 mole) and oxalyl chloride (0.2 moles) were mixed in benzene (80 ml) solution and kept at 20 C overnight. Unsaturated C_{20} acid chlorides were used after removal of solvent and reagent but C_6 and C_{11} acid chlorides were distilled.

Synthesis of Methyl 13-oxooctadecanoate-16,16-d_2 (1)

Hexanoyl-4,4-d_2 chloride was allowed to react with morpholinocyclododecene in the usual way (9). After conversion to methyl ester, b/0.1 mm 155-160 C, the yield of oxo ester was 66%.

Synthesis of Methyl 7-oxooctadecanoate-10,10-d_2 (2)

Reaction between dodecanoyl-4,4-d_2 chloride and morpholinocyclohexene was carried out as previously described (8) but the intermediate β-diketone was hydrolyzed with 8% NaOH in absolute ethanol as described for hydrolysis of β-diketones from the reaction with morpholinocyclododecene (9). The yield of oxo ester obtained after distillation and crystallization from methanol was 44%.

Synthesis of 8-oxooctadecanoate-11,11-d_2 (3) (15,16)

Concentrated H_2SO_4 (0.1 g) was added to a mixture of dry benzene (125 ml) and dihydropyran (0.345 moles dried over sodium and distilled immediately before use) at 15 C with stirring until the acid dissolved. Nonyl-2,2-d_2-malonic acid (0.115 moles) was added during 30 min at 20 C and the solution stirred for a further 1.5 hr. Potassium hydroxide pellets (4 g) were added, the mixture stirred for 30 min and, filtered through glass wool and benzene and excess dihydropyran was taken off at 25 C, leaving a thick liquid. Sodium (0.115 moles) was dispersed in xylene, transferred to benzene (250 ml) and the above malonate ester, in benzene (125 ml) was added. The mixture was stirred for 18 hr at 25 C under nitrogen when the sodium had reacted almost completely; 7-carbomethoxylheptanoyl chloride (0.115 moles) in benzene (100 ml) was then added over 15 min and the very thick mixture stirred for 4 hr. Acetic acid (12 ml) was added, the reaction mixture refluxed for 4 hr, cooled, and washed once with water; the solvent was

removed and the residue refluxed with 5% methanolic HCl (to break down polymerized dihydropyran) for 4 hr. After working up, the product was distilled giving the 8-oxo ester (0.09 moles, 78%); the b.p./0.15 mm was 160-165 C.

Synthesis of Methyl 11-oxooctadecanoate-14,14-d_2 (4)

This ester was prepared as above starting from hexyl-2,2-d_2-malonic acid and 10-carbomethoxydecanoyl chloride; the yield was 64%.

Synthesis of Methyl 11-oxooctadecanoate-8,8-d_2 (5)

Reaction between *cis*-11-eicosenoyl-4,4-d_2 chloride and hexylmalonic acid, as described above, gave crude 18-heptacosen-8-one-11,11-d_2 which was separated from dihydropyran polymerization products as the urea complex. The yield of ketone was 78% and the m.p. after crystallization from ethanol was 26-29 C. The ketone was epoxidized with *m*-chloroperbenzoic acid in CH_2Cl_2 for 6 hr, the crude epoxide refluxed with acetic acid and, after removal of acid, KOH hydrolysis gave the 18,19-diol. The diol was oxidized with lead tetraacetate in benzene for 1 hr at 25 C and the recovered aldehyde oxidized to 11-oxooctadecanoic-8,8-d_2 acid with chromic acid in acetic acid.

The crude acid, which contained some pelargonic acid and other by-products, was converted to methyl ester with 5% methanolic HCl. At this stage, a by-product, which appeared to be a saturated C_{27} ketone, crystallized from the methanol solution at room temperature; the weight was 12% of the weight of the original C_{27} ketone product. The methyl esters were chromatographed on Biosil A; elution with hexane-ether (98:2) gave methyl pelargonate and with hexane-ether (95:5) gave several fractions containing the 11-oxo C_{18} ester together with some C_{17}-C_{20} esters. GLC showed that the total oxo ester product would have the composition C_{17}, 3%; C_{18}, 88%; C_{19}, 2%; and C_{20}, 7%. Most of the C_{20} product was derived from 11-octadecenoic acid in the original oleic acid. Crystallization of the fractions from hexane gave a product with 96% C_{18} ester; the yield from 11-eicosenoic-4,4-d_2 acid was 30%.

Synthesis of Methyl 8-oxooctadecanoate-5,5-d_2 (6)

18-Triaconten-11-one-14,14-d_2 was prepared from nonylmalonic acid and *cis*-8-eicosenoyl-4,4-d_2 chloride as above; the m.p. was 38-41 C and the yield 73%. Oxidative cleavage and purification as before then gave the dideuterated 8-oxo ester in 26% yield from C_{20} acid; the GLC composition was C_{16}, 1%; C_{17}, 3%; C_{18}, 94%; and C_{19}, 2%.

Synthesis of Methyl 12-oxooctadecanoate-9,9-d_2 (7)

18-Nonadecen-7-one-10,10-d_2 was prepared from 12-tridecenoyl-4,4-d_2 chloride and pentylmalonic acid as described above. Crude product was saponified and neutral ketonic products (and polymerization by-products) were extracted. Distillation (b.p./0.05 mm was 125-130 C) followed by crystallization from MeOH, gave the dideuteroketone; yield was 50% and m.p. was 36-38 C. Oxidation as before gave the 12-oxo ester in 26% yield from C_{13} acid. GLC showed that when only one D_2O exchange had been carried out on the original C_{11} acid, the dideuterated 12-oxo ester contained only 1% of C_{17} acid, but when two exchanges had been performed, about 11% of C_{17} acid was present. In this case, product was purified by hexane crystallization.

Reduction of Oxo Esters to Dideuterooctadecanoates

The above seven oxo esters were reduced as tosylhydrazones as described by Hutchins et al. (17); after purification by silicic acid column chromatography and distillation the yields of dideuterooctadecanoates ranged from 52 to 67% (average yield 59%).

RESULTS AND DISCUSSION

To synthesize the *gem* dideuterooctadecanoates, a good synthesis of 2,2-dideutero acids was first required. Therefore, the α deuteration of C_2 to C_4 acids by exchange effected by heating the sodium or potassium salt in 0.05 molar OD^- at 150 C, as reported by Atkinson et al. (18), was investigated. When this procedure was applied to sodium palmitate, the reaction was very slow and incomplete; thus, after 18 hr at 150 C (with a 50 times molar excess of D_2O containing 1% NaOD), exchange was only about 35% complete. However, exchange was practically complete at 200 C, and nine 2,2-dideutero acids from C_4 to C_{18} were prepared in yields of over 80% (Table I).

Since double bond migration occurs when unsaturated acids are heated in strong alkali (19), attempts were first made to deuterate sodium oleate at 150 C. After 18 hr at 150 C, the product contained 40% deutero and 30% dideutero acids, and after 3 further exchanges the reaction was still only 80% complete; therefore, reaction was subsequently always carried out at 200 C. ^{13}C NMR indicated that appreciable double bond migration had not occurred (2). The double bond of 10-undecylenic acid readily migrates into the 9,10 position in alkali (20) but ^1H NMR indicated that this occurred to only a minor extent in the present reaction after one D_2O exchange. To lessen the possi-

TABLE I

Yields and Isotopic Purity of 2,2-dideutero Acids

Acid	Exchange conditions[a]	Yield (%)[b]	Isotopic purity (by MS)	
			D_2	D
Butyric	C	88	99	1
Hexanoic	B	81	98	2
Nonanoic	B	89	99	1
Decanoic	B	84	97	3
10-Undecenoic	A	86	c	c
Hexadecanoic	A	95	98	2
Octadecanoic	A	91	98	2
6-Octadecenoic	A	93	97	3
9-Octadecenoic	A	90	98	2

[a]Exchange conditions were: A, 0.1 mole Na salt in 100 ml D_2O containing 1% NaOD, one exchange only for 3 days; B, 0.25 mole Na salt in 100 ml D_2O containing 1% NaOD, two exchanges for 3 days each; C, as in B but four exchanges for 2 days each: temperature was 200 C in each reaction.

[b]Product after distillation.

[c]Could not be measured by MS due to presence of unusually large M+1 and M+2 peaks in both labeled and unlabeled esters but ^1H NMR indicated that exchange was about 95% complete.

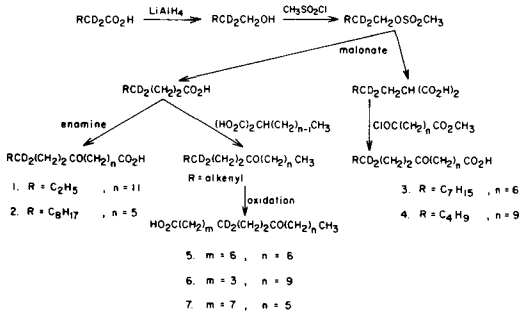

FIG. 1. Syntheses of dideuterated oxooctadecanoic acids with the deuterated carbon in the position γ to the oxo group.

bility of double bond migration in unsaturated acids, exchange was also carried out in 0.5% NaOD (instead of 1%) without affecting the rate of exchange.

Chain extension was then used to prepare dideuterooctadecanoic acids from products listed in Table I. Octadecanoic-4,4-d_2 acid was prepared by anodic coupling of hexadecanoic-2,2-d_2 acid with methyl hydrogen succinate but the yield was only 19%. Relatively low yields and formation of by-products are major disadvantages of fatty acid synthesis by electrolytic coupling (21). For this reason, other methods of chain extension were chosen which would yield intermediate dideuterated oxo acids which could then be reduced to the required dideuterooctadecanoates. Three chain lengthening routes are shown in Figure 1. The dideuterated oxo acids were required to have both deuterium atoms attached to carbon in the position γ to the oxo group so that they would have maximum effect on the ^{13}C NMR spectra, and it was also necessary to separate CD_2 and oxo groups so that deuterons would not be lost during synthesis.

Each 2,2-dideutero acid intermediate was lengthened, using the malonate synthesis described by Spener and Mangold (14), to give either 4,4-dideutero acid or the 2,2-dideuteroalkylmalonic acid; yields were 57 to 85% (from 2,2-dideutero alcohol). Dideuterated oxo acids with the deuterated carbon on the terminal CH_3 side of the oxo group were obtained by two routes. First, application of the enamine synthesis (8,9) gave 13-oxooctadecanoic-16,16-d_2 acid (1) (from hexanoyl-4,4-d_2 chloride and morpholinocyclododecene) and 7-oxooctadecanoic-10,10-d_2 acid (2) (from dodecanoyl-4,4-d_2 chloride and morpholinocyclohexene). Secondly, reaction of 2,2-dideuteroalkylmalonic acids with half ester acid chlorides using the ketone synthesis of Bowman and Fordham (15), gave 8-oxooctadecanoic-11,11-d_2 acid (3) and 11-oxooctadecanoic-14,14-d_2 acid (4).

Dideuterated oxo acids with the deuterated carbon on the carboxyl side of the oxo group were prepared starting from 4,4-dideuterated unsaturated acids, the double bond acting as a potential carboxyl group. Reaction of the three 4,4-dideuterated monoenoic acid chlorides with appropriate alkylmalonic acids gave dideutero C_{27},C_{30}, and C_{19} ketones each with an 18,19 double bond (Fig. 1). Stepwise oxidative cleavage of the double bond by epoxidation, hydrol-

TABLE II

Yields and Isotopic Purity of Methyl Dideuterooctadecanoates

Position of CD_2 group	Yield[a]	Isotopic purity	
		D_2(M+ = 300)	D(M+ = 299)
2	91	98	2
3	80	99	1
4	19	98	2
5	11	97	3
6	55[b]	97	3
7	60[b]	97	3
8	12	97	3
9	11	96	4
10	17	97	3
11	21	99	1
12	45[b]	96	4
13	57[b]	97	3
14	24	98	2
15	36	97	3
16	26	99	1
17	58	97	3

[a]Overall yields calculated from unlabeled starting material.

[b]Yields recalculated to include product obtainable from recovered alcohol.

ysis to the diol, cleavage to the aldehyde, and finally oxidation to the acid gave 11-oxooctadecanoic-8,8-d_2 **(5)**, 8-oxooctadecanoic-5,5-d_2 **(6)**, and 12-oxooctadecanoic-9,9-d_2 **(7)** acids. GLC showed that acids of other chain lengths (C_{16}-C_{19}) resulting either from over oxidation or double bond migration, during reaction in $NaOD/D_2O$, did not exceed about 6% of the product for **5** and **6** but formed a greater percentage of the product **7** when more than one D_2O exchange was used at the beginning of the synthesis. Further dideuterated oxo acids of this type could also be prepared in this way using other natural or synthetic monoenoic acids.

The seven acids with two deuterons at carbons 5-, 8-, 9-, 10-, 11-, 14-, and 16- were then prepared by reducing tosylhydrazones of the esters from the above oxo acids with sodium cyanoborohydride (17). Octadecanoic-3,3-d_2 acid was also prepared by chain extension by the malonate synthesis from hexadecan-1-ol-1,1-d_2 obtained by reducing methyl palmitate with lithium aluminum deuteride.

Since certain oxooctadecanoic acids can be readily derived from natural sources, or easily synthesized, it was convenient to prepare the corresponding dideutero acids from them by stepwise reduction of the oxo group. The six acids with deuteriums at carbons 6-, 7-, 12-, 13-, 15-, and 17- were prepared by this route which was previously employed to prepare octadecanoic-17,17-d_2 acid (22). In this study, the method was reinvestigated and overall yields improved. The 6-, 7-, and 13-oxooctadecanoates were obtained by enamine synthesis (7-9), 12-oxooctadecanoate by oxidation of hydrogenated castor oil esters, and 17-oxooctadecanoate from 17-hydroxyoctadecanoate (10). To prepare octadecanoic-15,15-d_2 acid, hexadecanoic-13,13-d_2 acid was first prepared from 13-oxohexadecanoic acid and the chain lengthened by malonate synthesis.

Oxo esters were reduced with $NaBD_4$ to deutero hydroxy esters in high yields and three of the hydroxy esters were converted to tosylates and three to mesylates. Tosylates were prepared in pyridine and required a fivefold excess of tosyl chloride, yields were 70-80%. The mesylate preparation, a modification of the procedure of Crossland and Servis (11), was simpler since pyridine was not employed as a solvent; yields were 80-90%. The melting points were, however, lower than those of comparable tosylates.

Both mesylates and tosylates were reduced with $LiAlD_4$ to tetradeutero alcohols which were oxidized to dideutero acids with chromium trioxide in acetic acid. About 50% of the product consisted of C_{36} ester probably formed by hemiacetal oxidation (23). Oxidation was also carried out at greater dilution, in acetic acid containing 10% water and with CrO_3/H_2SO_4 in acetone solution (24), but ca. 30% of long chain ester was still formed. Methanolysis of the oxidation product gave dideuterated esters and alcohol from the C_{36} ester which, along with a number of by-products from the reduction, were separated by column chromatography. Average overall yield from oxo ester, using tosylate, was 43% and, using mesylate, was 46%; however, when

recovered alcohol was reoxidized, these percentages rose to 57 and 60%, respectively. There was not much difference between the two methods because, though yield of mesylate from hydroxy ester was better, more by-products were formed during mesylate reduction than during tosylate reduction.

When the synthetic routes are compared (Table II), yields of octadecanoic-2,2-d_2 and -3,3-d_2 acids are naturally the highest since the syntheses are most direct. Synthesis by converting CO to CD_2 is the next best method but is limited to easily obtainable oxo esters. Other oxo esters could be prepared by the lengthier ketone synthesis (15), but starting materials are less readily available. In this study, seven dideutero esters were prepared by reduction of dideutero oxo esters, but yields were lower in these longer syntheses. Reduction of dideutero oxo acids by the Wolf Kishner reaction was not investigated since yields of chromatographically pure products from this reaction often do not exceed 70%. Also, it was felt that a milder method was preferable for deuterated compounds.

Table II shows that all products were obtained with high isotopic purity. Isomers prepared by converting CO to CD_2 do not exceed 97% dideutero ester since the $NaBD_4$ was 98% D_4 and the $LiAlD_4$ was 99% D_4. Octadecanoate-3,3-d_2, however, was 99% dideutero ester because only one reduction with $LiAlD_4$ was employed.

The other isomers have the same isotopic purity as the 2,2-dideutero acids from which they were prepared, showing that no loss of deuterium occurred during synthesis. High isotopic purity is, therefore, best obtained by repeated exchange with D_2O at the first stage as in the preparation of butyric-2,2-d_2 acid which was the starting material for synthesis of octadecanoic-16,16-d_2 acid. All the syntheses reported here can be reasonably conveniently used for larger scale preparation, and even the longest ones have overall yields of over 10%.

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Autoxidation of Methyl Linoleate. Separation and Analysis of Isomeric Mixtures of Methyl Linoleate Hydroperoxides and Methyl Hydroxylinoleates

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ABSTRACT

The mixture of conjugated diene hydroperoxide isomers obtained from autoxidation of methyl linoleate was separated by high performance liquid chromatography (HPLC). Four major isomers were obtained from adsorption chromatography and identified as the 9 and 13 positional isomers having the *trans-trans* and *cis-trans* configurations. The latter geometrical isomers have the *trans* double bond adjacent to the hydroperoxide group. The hydroxy compounds (methyl hydroxylinoleates) obtained from the hydroperoxides by $NaBH_4$ reduction were similarly separated but with improved resolution. This is the first instance of the complete separation of these compounds and provides a rapid method for their analysis. Unlike adsorption chromatography, reversed-phase chromatography separates the mixtures only according to the geometrical isomerism of the double bonds and not according to the position of the hydroxy or hydroperoxide function.

INTRODUCTION

In a previous communication (1), we reported the separation and analysis, by high performance liquid chromatography (HPLC), of the isomers of linoleate hydroperoxide methyl esters obtained from oxidations of linoleic acid catalyzed by lipoxygenases. The separations of hydroperoxide isomers obtained from lipoxygenase oxidation of linoleic acid (2) and those of arachidonate and γ-linolenate (3) have since been reported. All these procedures take advantage of the specificities of lipoxygenases so that the mixtures to be analyzed already contain a preponderance of a single isomeric species. The mixture of hydroperoxide isomers obtained from autoxidation of linoleate is, however, considerably more complex. It is now generally accepted (4-8) that only two positional isomers, 9 and 13, are formed to any appreciable extent. However, each positional isomer can be present as a mixture of a maximum of four (*cis-cis*, *trans-trans* and 2 *cis-trans*) geometric isomers. Previous studies in the distribution of isomers derived from autoxidation have been based on obtaining concentrates containing mainly *cis-trans* or *trans-trans* isomers of the hydroperoxides and the corresponding hydroxy compounds (hydroxylinoleates) and analysis of their infrared (IR) spectras (6-8). This technique is inherently open to errors in quantitative determinations and is also dependent on the reference standard chosen (8). Although IR spectroscopy is useful in determining the purity of a *trans-trans* isomer of which a highly purified sample has been obtained (9), it is not reliable in determining minor amounts of *trans-trans* isomers in a mixture. It also does not distinguish between the two types of *cis-trans* isomers nor does it allow simultaneous analysis of the distribution of geometric and positional isomers. It is apparent that the chromatographic separation of the individual isomers is desirable if more effective analysis is to be achieved. To this end, we have improved the original chromatographic procedure for the separation of linoleate hydroperoxide isomers by using finer (5 micron instead of 10 micron) silica particles and a larger (0.7 cm instead of 0.46 cm diameter) column. This has resulted in the separation, as single components, of the isomers of the hydroperoxides obtained from the autoxidation of methyl linoleate as well as the methyl hydroxylinoleates derived (by $NaBH_4$ reduction) from them. The identification of the components resolved by the improved chromatographic procedure as well as the chromatography of the isomeric mixtures on a reversed-phase system is now reported. As the pure isomers of linoleate hydroperoxides readily undergo isomerization (10) thereby possibly interfering with previous analysis, the properties of individual isomers of defined isomeric purity is also discussed.

MATERIALS AND METHODS

Methyl linoleate (99%) was obtained from Lipid Supplies (St. Andrews, U.K.), Hiflosil from Applied Sciences Labs, Inc. (State College, PA), Partisil-5 and Partisil-ODS from Whatman (Maidstone, U.K.).

Ultraviolet (UV) spectra were recorded in

ethanol solution on a Pye-Unicam SP800 and IR spectra (in CCl_4 solution) on a Pye-Unicam SP200G spectrophotometer. Nuclear magnetic resonance (NMR) spectra were recorded on a Perkin Elmer R32 (90 MHz) spectrometer.

High Performance Liquid Chromatography (HPLC)

Adsorption chromatography was carried out on a column (50 cm x 0.7 cm) of Partisil-5 packed by a balanced density slurry method (11). The column was maintained at 30 C by a water jacket and solvent was equilibrated to this temperature via a heat exchanger consisting of a coil of stainless steel tubing (200 cm x 0.16 cm) placed inside a water jacket which was fixed immediately before the injection head. Solvent flow-rate through the column was maintained at 4 ml/min by a Waters 6000 pump, and a Cecil 212 variable wavelength UV detector was used to monitor the effluent at 234 nm via a 10 μl (1 cm) flow-cell. The eluting solvent consisted of 0.75% anhydrous ethanol in hexane. The hexane had been purified by percolating through a sulfuric acid column (12) followed by a twin-bed column of basic (15 cm) and neutral (15 cm) alumina and then distilled via a fractionating column (100 cm) of of Fenske helices.

Reversed-phase chromatography was carried out at ambient temperature on a column (25 cm x 0.46 cm) of Partisil-ODS (octadecylsilane bonded Partisil-10). The eluting solvent (ethanol/water 60:40) was maintained at a constant flow-rate of 0.6 ml/min.

For the isolation of individual components, 1-2 mg of the mixture of methyl linoleate hydroperoxides or hydroxylinoleates were repeatedly injected and the components collected. Injecting larger quantities caused peak-broadening and hence loss of resolution. To recover compounds separated by chromatography on Partisil-ODS, an equal volumn of water was added to the collected components which were then extracted with hexane (3X original volume).

Preparation of Methyl Linoleate Hydroperoxide Isomers

Methyl linoleate (2 g) was autoxidized in air, in a Warburg apparatus at 30 C, in the dark until oxidation (as measured by oxygen-uptake) had reached ca. 5% (48-72 hr). The contents of the Warburg flask were dissolved in hexane (40 ml) and the mixture of hydroperoxides isolated by the "counter-current" method of Zilch et al. (13). This mixture was then purified by chromatography on a column (20 cm x 1.5 cm) of Hiflosil. Stepwise elution used 100 ml hexane-diethyl ether (90:10) followed by hexane-diethyl ether (80:20). The hydroperoxide-containing fractions (ferrous thiocyanate spot test) were pooled.

Methyl 9-D-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate and methyl 13-L-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate were prepared by lipoxygenase oxidation of linoleate as previously described (14).

Reduction to Hydroxylinoleate and Hydroxystearate

The linoleate hydroperoxides were reduced, as a mixture or as individual components, to the corresponding hydroxylinoleates by $NaBH_4$ in methanol and to the hydroxystearates by hydrogenation as previously described (14).

RESULTS

Separation of Methyl Linoleate Hydroperoxide Isomers by Chromatography on Partisil-5

Figure 1 shows the separation of the mixture of methyl linoleate hydroperoxides into four (I-IV) major components. The improved efficiency achieved by using 5 micron particles is indicated by the number of effective theoretical plates per meter (21,000) for hydroperoxide isomers which is an order of magnitude higher than that obtained with the 10 micron particles (N_{eff} = 2000)* previously used (1,10). This is reflected by a better separation of the component and more importantly, by the separation of components III and IV which were not resolved previously. The four major components constitute 97% of diene-hydroperoxides present in the chromatogram. The minor components in the region of the chromatogram containing the diene-hydroperoxides are possibly other linoleate hydroperoxide isomers but have not been analyzed owing to the difficulty of isolation in a high degree of isomeric purity and of the tendency to isomerize (10) into the other isomers. This has also hampered the spectroscopic analysis of the pure major isomers as isomerization occurs readily especially in the high concentrations used for IR and NMR spectra. Unless great care is taken to limit the duration in which the isomers are kept at high concentration, 5-10% and in some cases

*The numbers of effective theoretical plates (N_{eff}) were calculated using the formula $N(eff) = 16\,[(tr - to)/w]^2$ where

tr = retention time of component,
to = retention time of a component not retained, i.e. dead volume of column,
w = width of the component peak at its base.

The N_{eff} values were calculated using the oxygenated fatty acid components in the chromatogram rather than standard mixtures of hydrocarbons which yielded higher values.

20% isomerization can occur. In the following analysis of the pure components, the isomeric purity of each component was determined by HPLC both before and after each spectral analysis and in each case the isomeric purity was > 90%.

Position of the Hydroperoxide Group

The mass spectra of the hydroxystearates derived from the reduction of the individual components showed that I and II are 13- and III and IV are 9-positional isomers.

Infrared Spectra

The IR of components I and III contained two absorption bands at 949 and 986 cm^{-1} (*cis-trans*), and those of components II and IV had a single band 989 cm^{-1} (*trans-trans*).

Nuclear Magnetic Resonance Spectra

The NMR spectrum of component I is identical to that of methyl 13-L-hydroperoxy-*cis*-9-*trans*-11 octadecadienoate obtained from soybean lipoxygenase oxidation. Similarly, the spectrum of component III is identical to that of methyl 9-D-hydroperoxy-*trans*-10-*cis*-12-octadecadienoate obtained from potato lipoxygenase reaction. This establishes that the *cis-trans* configuration in components I and III has the *trans* double bond adjacent to the hydroperoxide group rather than the alternative with the *cis* double bond adjacent to it. The identities of components I and III were further established by co-injection of the single isomers obtained from lipoxygenase reactions with the mixture obtained from autoxidation. The components I and III cochromatographed with the lipoxygenase-generated 13- and 9-*cis-trans* isomers, respectively. From the above data the four components may be assigned the following structures:

I, Methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate
II, Methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate
III, Methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate
IV, Methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate

The NMR spectra of the 9- and 13-*cis-trans* isomers are indistinguishable as evidenced by the partial spectra (Fig. 2a and b) of the '9-hydroperoxide methyl ester' obtained from potato lipoxygenase reaction and of component I from autoxidation. While the olefinic protons of the *cis-trans* isomers of both the hydroperoxide and hydroxy compounds can be subject to analysis (15,16) as first order spectra, the

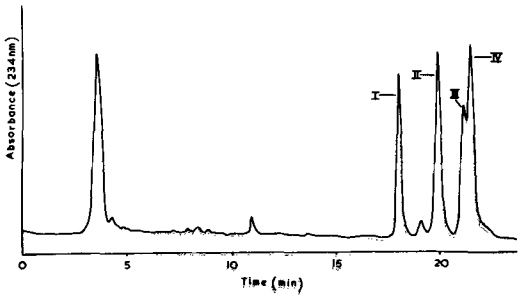

FIG. 1. High performance liquid chromatography (HPLC) of methyl linoleate hydroperoxide isomers; I, methyl 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoate; II, methyl 13-hydroperoxy-*trans*-9, *trans*-11-octadecadienoate; III, methyl 9-hydroperoxy-*trans*-10, *cis*-12-octadecadienoate; IV, methyl 9-hydroperoxy-*trans*-10, *trans*-12-octadecadienoate.

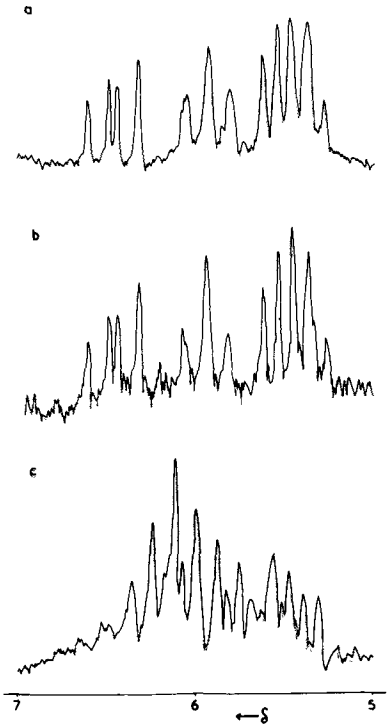

FIG. 2. Nuclear magnetic resonance (NMR) spectra of methyl linoleate hydroperoxides. (olefinic protons); (a), methyl 9-D-hydroperoxy-*trans*-10-*cis*,12-octadecadienoate; (b), Component I; (c) Component II. Spectra were accumulated using a Digital Signal Averager from 16 (5 min) scans of a solution of the hydroperoxides (1-2 mg) in CCl_4 solution.

corresponding protons of the *trans-trans* isomers (represented by the spectrum of component II in Figure 2c) are too closely grouped together to be so analyzed. Even with the allylic protons decoupled, a complex ABCD system remains.

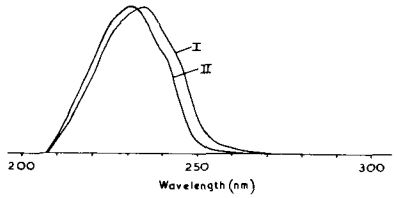

FIG. 3. Ultraviolet (UV) spectra (EtOH) of Components I and II.

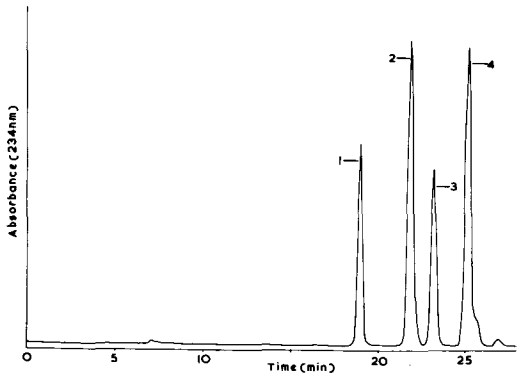

FIG. 4. High performance liquid chromatography (HPLC) of methyl hydroxylinoleate isomers; 1, Methyl 13-hydroxy-*cis*-9, *trans*-11-octadecadienoate; 2, methyl 13-hydroxy-*trans*-9, *trans*-11-octadecadienoate; 3, methyl 9-hydroxy-*trans*-10, *cis*-12-octadecadienoate; 4, methyl 9-hydroxy-*trans*-10, *trans*-12-octadecadienoate.

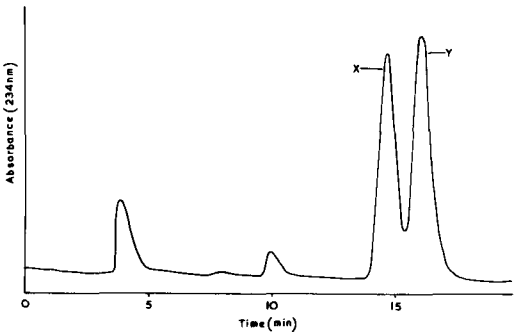

FIG. 5. Reversed-phase high performance liquid chromatography (HPLC) of methyl linoleate hydroperoxide isomers; X, mixture of *cis-trans* isomers (i.e. components I & III in Fig. 1); Y, mixture of *trans-trans* isomers (i.e. components II & IV in Fig. 1).

Ultraviolet Spectra

The UV spectrum (Fig. 3) in ethanol solution of a *cis-trans* isomer (component I) is bathochromically shifted (λmax = 236 nm) from that of a *trans-trans* isomer (component II, λmax = 233 nm). This is directly analogous to the bathochromic shift observed in *cis-trans* and *trans-trans* isomers of conjugated fatty acids (17-19) upon which previous discussions concerning conjugated linoleate hydroperoxides were largely based (5,6,8,20). The direct observation of the differences is absorption maxima between the two geometrical isomers of methyl linoleate hydroperoxides is a result of the high isomeric purity (>93%) of the components obtained from HPLC since coalescence of the maxima occurs in mixtures of the two geometrical isomers.

The apparent molar absorbances of the components based on iodometric estimation (21) of the peroxide content of a solution of the pure isomers were as follows: I ϵ_{236} = 26,000; II ϵ_{233} = 28.600; III ϵ_{236} = 25,900; and IV ϵ_{233} = 28,600. These are broadly in agreement with previous results (5,6,8,9.20) and in particular, the higher values of the *trans-trans* compared with the *cis-trans* isomers are similar to those observed in the conjugated fatty acid series (17-19). However, the molar absorbance values obtained above are subject to possible inaccuracies in the estimation of the peroxide content of a solution. Although the usual (gravimetric) method of determining molar absorbance values may be applied to the hydroxylinoleates (see below), consistent values cannot be obtained by this method for the single components of hydroperoxides due, presumably, to their decomposition to volatile compounds.

Chromatography of Methyl Hydroxylinoleate Isomers

The mixture of methyl hydroxylinoleate isomers obtained by sodium borohydride reduction of the mixture of methyl linoleate hydroperoxides is resolved into four major components by chromatography on Partisil-5 (Fig. 4). The resolution is superior to that achieved for the hydroperoxides. This improvement is due to an increased efficiency (N_{eff} = 35,000) as well as to a shift in the relative retention times of the components. A combination of UV and IR spectra of the hydroxylinoleates and mass spectra of the hydroxystearates derived from them established the structure of the components shown in Figure 4 as follows:

1, Methyl 13-hydroxy-*cis*-9,*trans*-11-octadecadienoate
2, Methyl 13-hydroxy-*trans*-9,*trans*-11-octadecadienoate
3, Methyl 9-hydroxy-*trans*-10,*cis*-12-octadecadienoate
4, Methyl 9-hydroxy-*trans*-10,*trans*-12-octadecadienoate

This was confirmed by reduction of the individual components of hydroperoxides which showed that I gives rise to 1, II to 2, III to 3,

and IV to 4. In addition, reduction of the 9- and 13-isomers of hydroperoxide from lipoxygenase oxidations showed that methyl 9-D-hydroxy-*trans*-10, *cis*-12-octadecadienoate and methyl 13-L-hydroxy-*cis*-9,*trans*-11-octadecadienoate co-chromatographed with components 3 and 1, respectively. The molar absorbances and absorption maxima (in ethanol) of the components were: 1, λmax, 236 nm, ϵ = 27,200; 2, λmax, 233 nm, ϵ = 30,500; 3, λmax, 236 nm, ϵ = 28,300; 4, λmax, 233 nm, ϵ = 31,600. Using these molar absorbance values, the percentage compositions of the four hydroxylinoleate components calculated from duplicate HPLC chromatograms of two separate autoxidation preparations were: 1, 20.1 ± 0.9; 2, 28.9 ± 1.8; 3, 21.3 ± 2.4; 4, 29.7 ± 1.4. This shows that the proportions of 9 (components 3 and 4) and 13 (components 1 and 2) positional isomers formed are (within experimental error) equal.

Reversed-Phase Chromatography on Partisil-ODS

The mixture of hydroperoxide isomers were resolved into two components X and Y (Fig. 5) upon chromatography on octadecylsilane bonded 10 micron silica particles (Partisil-ODS). Component X contained *cis-trans* isomers (λmax = 236 nm, νmax, 949,986 cm^{-1}) and component Y *trans-trans* isomers (λmax = 233 nm, νmax, 989 cm^{-1}). The fact that both components contained a mixture of 9- and 13-positional isomers was confirmed by rechromatography on Partisil-5 when component X gave rise to components I and III and Y to II and IV. In addition, both lipoxygenase-produced 9- and 13- isomers cochromatographed with component X.

Thus, unlike adosprtion chromatography on Partisil-5, reversed-phase chromatography separated the hydroperoxide methyl esters only according to the geometrical isomerism of the double bonds and not according to the position of the hydroperoxide group. This is also the case for the reversed-phase chromatography of the hydroxy compounds which were resolved into the *cis-trans* and *trans-trans* mixtures with the same order of elution as found for the hydroperoxides.

DISCUSSION

The results of this investigation show that the primary products of autoxidation, i.e. hydroperoxides, can be analyzed and handled as distinct isomers by the technique of HPLC. Milligram quantities of the individual isomers may be prepared and studied. When the prime objective is not the investigation of the properties of the hydroperoxides, but the distribution of the isomers in the oxidation of linoleate, then the procedure of conversion to the corresponding hydroxy compounds and analyzing them by HPLC may be used to advantage because of the superior resolution and the fact that, unlike the hydroperoxides, they do not isomerize under the conditions used (10).

The separation of the methyl hydroxyoctadecadienoates also means that the analysis of mixtures of these compounds in oilseeds, hitherto unresolved by a one-step chromatographic procedure (22), may now be conducted rapidly.

The chromatographic behavior of the hydroxy compounds follows closely that of the hydroperoxides. In adsorption chromatography, the 13-isomer is eluted before the 9-isomer and the *cis-trans* before the *trans-trans* isomer in both cases.

Autoxidation under the conditions employed in this investigation produces only four major components out of a possible total of eight isomers having the hydroperoxide group at positions 9 and 13. The *cis-cis* isomers and perhaps more significantly, the *cis-trans* isomers with the *cis* double bond adjacent to the hydroperoxide group are not formed to any appreciable extent. Although the absence of these isomers has been assumed in previous work in autoxidation, it is only with the chromatographic separation of isomers that this has now been established. The distribution of isomers reported here may have significant implications in the mechanism of reactions of molecular oxygen with diene-radicals. However, since the conditions of autoxidation used are those in which the hydroperoxide isomers undergo interconversion (10), the distribution of isomers may reflect the equilibrium mixture under those conditions rather than any specificity in the formation of hydroperoxides. The variation in the proportion of *cis-trans* and *trans-trans* isomers with the temperature of autoxidation has previously been observed (6,7). The distribution of isomers under different conditions of autoxidation and the possible role of the isomerization reaction in affecting the distribution is currently under investigation.

In a previous report (10), the role of the isomerization reaction in affecting the distribution of isomers obtained from lipoxygenase oxidation of linoleate was discussed. In that investigation, the methyl linoleate hydroperoxide isomers were only resolved into three components. The two 9-isomers, here resolved as components III and IV, were not resolved by using 10 micron particles but appear as a single

component (component 3). The isomerization of all four major isomers has now been studied (Chan & Levett, in preparation) using the improved chromatographic system discussed above and will be reported elsewhere.

ACKNOWLEDGMENTS

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Radical Scavenging Reactions of α-Tocopherol II. The Reaction with Some Alkyl Radicals

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ABSTRACT

α-Tocopherol was reacted with some alkyl radicals (ethyl, n-propyl, iso-propyl, n-butyl, and sec-butyl radical) to study its radical scavenging reactivity. The two types of products (alkyl ethers of α-tocopherol and cyclohexadienones) were obtained on treatment of each radical. These structures were determined by the spectral analysis. It was observed that α-tocopherol is very sensitive to the alkyl racidals and that the yields of the cyclohexadienones are decreased and that of the alkyl ethers are not much varied with an increase of carbon numbers of the alkyl radicals.

INTRODUCTION

There is considerable evidence that free radicals attack polyunsaturated fatty acids in cell membranes in vivo and that the degradation of these fatty acids results in cell damage (1,2). It is considered that vitamin E would be one of the most effective compounds for protection against these cell impairments (3). Thus, the elucidation of radical scavenging mechanism of tocopherol is extremely important. Recently, we reported on a radical scavenging reaction of α-tocopherol with methyl radical, which afforded two major products of (IIa) and (IIIa) (4). The present study was undertaken to confirm whether the radical scavenging reactivity mentioned above was generalized as a nature of α-tocopherol for common alkyl radicals.

MATERIALS AND METHODS

dl-α-Tocopherol was purchased from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Diethyl, di-n-propyl, di-iso-propyl, di-n-butyl and di-sec-butyl sulfoxide are synthesized by the oxidation of the corresponding dialkyl sulfides with sodium periodate in water at 5 C (5). All other chemicals were obtained from common laboratory suppliers. Each radical was generated from the corresponding dialkyl sulfoxide and reacted with α-tocopherol by a similar method described previously, except sec-butyl radical (4). The latter was reacted at about 110 C because of its higher melting point. The reaction mixtures were purified by silica gel column chromatography, in which a mixture of n-hexane and benzene (5:1 v/v) was used as an elution solvent. Each fraction collected was analyzed by gas liquid and thin layer chromatography.

Mass, UV, IR, ^{1}H-NMR, and ^{13}C-NMR spectra were taken by the methods mentioned in the previous paper (4).

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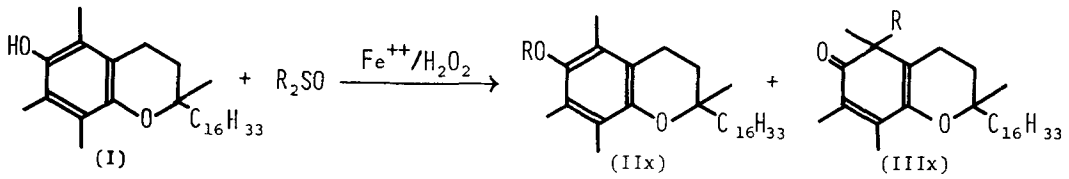

R = Methyl -------- (IIa), (IIIa)
Ethyl --------- (IIb), (IIIb)
n-Propyl ------ (IIc), (IIIc)
iso-Propyl ---- (IId), (IIId)
n-Butyl ------- (IIe), (IIIe)
sec-Butyl ----- (IIf), (IIIf)

FIG. 1. The radical alkylation of α-tocopherol.

TABLE I

The Spectral Data of IIb-f

	IIb	IIc	IId	IIe	IIf
MASS (M^+)	458	472	472	486	486
UV λ_{max}^{hexane} nm (ϵ)	280 (2700) 286 (2800) 290 (2600)	280 (2800) 286 (2500) 290 (2200)	283 (2500) 288 (2700) 291 (2500)	280 (2600) 286 (2900) 291 (2900)	280 (3000) 286 (3500) 289 (3400)
IR ν_{max}^{neat} cm^{-1}	1040	1010	1015	1020	1020
^{1}H-NMR[a] $\delta_{TMS}^{CDCl_3}$	3.72 (q^b,2H,J=6.5Hz)	3.58 (t,2H,J=6.5Hz)	3.94 (m,1H)	3.62 (t,2H,J=6.5Hz)	3.40 (d,2H,J=6.0Hz)
^{13}C-NMR[a] $\delta_{TMS}^{CDCl_3}$	– – 15.6 (q) 68.4 (t) 31.4 (s) 117.3 (s) 122.7 (s) 125.7 (s) 127.8 (s) 147.5 (s) 148.5 (s)	– 10.7 (q) 23.5 (t) 74.5 (t) 31.4 (s) 117.3 (s) 122.6 (s) 125.7 (s) 127.7 (s) 147.5 (s) 148.3 (s)	– – 22.4 (q) 74.9 (d) 31.5 (s) 117.3 (s) 122.7 (s) 126.3 (s) 128.3 (s) 147.2 (s) 147.3 (s)	14.0 (q^b)[c] 19.5 (t) 32.5 (t) 72.8 (t) 31.4 (s) 117.4 (s) 122.7 (s) 125.8 (s) 127.8 (s) 147.6 (s) 148.5 (s)	– 19.5 (q) 29.3 (d) 79.3 (t) 31.4 (s) 117.4 (s) 122.8 (s) 125.7 (s) 127.8 (s) 147.6 (s) 148.2 (s)

[a]Only typical signals are cited in part.

[b]The following abbreviations are used: d;doublet, t;triplet, q;quartet, m;multiplet.

[c]Letters in parentheses show the multiplicity of signals in the off-resonance experiment.

TABLE II

The Spectral Data of IIIb-f

	IIIb	IIIc	IIId	IIIe	IIIf
MASS (M^+)	458	472	472	486	486
UV λ_{max}^{hexane} nm (ϵ)	337 (3300)	336 (3200)	337 (2600)	336 (2500)	338 (3600)
IR ν_{max}^{neat} cm^{-1}	1635 1585	1632 1580	1635 1585	1630 1585	1635 1582
^{1}H-NMR[a] $\delta_{TMS}^{CDCl_3}$	1.86 (s[b],3H) 2.00 (s,3H) 2.12 (t,2H,J=6.5Hz)	1.86 (s,3H) 2.01 (s,3H) 2.12 (t,2H,J=6.5Hz)	1.83 (s,3H) 1.99 (s,3H) 2.14 (t,2H,J=6.5Hz)	1.85 (s,3H) 2.00 (s,3H) 2.11 (t,2H,J=6.5Hz)	1.86 (s,3H) 2.01 (s,3H) 2.14 (t,2H,J=6.5Hz)
^{13}C-NMR[a] $\delta_{TMS}^{CDCl_3}$	– – 9.6 (q) 11.2 (q) 14.3 (q) 18.2 (t) 51.5 (s) 74.9 (s) 116.3 (s) 128.9 (s) 142.5 (s) 147.4 (s) 204.5 (s)	– 11.2 (q) 14.3 (q) 14.5 (q) 18.6 (q) 41.9 (t) 51.0 (s) 74.9 (s) 116.9 (s) 128.6 (s) 142.4 (s) 147.5 (s) 204.5 (s)	– 11.1 (q) 14.2 (q) 16.7 (q) 19.2 (q) 22.5 (d) 53.5 (s) 74.9 (s) 117.2 (s) 129.9 (s) 143.1 (s) 147.1 (s) 204.5 (s)	11.2 (q[b])[c] 14.2 (q) 14.3 (q) 23.1 (t) 26.9 (t) 39.2 (t) 51.0 (s) 74.9 (s) 116.8 (s) 129.6 (s) 142.5 (s) 147.5 (s) 204.5 (s)	11.4 (q) 14.3 (q) 23.7 (q) 24.2 (q) 28.5 (d) 48.6 (t) 50.5 (s) 75.0 (s) 117.5 (s) 128.7 (s) 142.3 (s) 147.0 (s) 204.1 (s)

[a]Only typical signals are cited in part.

[b]The following abbreviations are used: d;doublet, t;triplet, q;quartet, m;multiplet.

[c]Letters in parentheses show the multiplicity of signals in the off-resonance experiment.

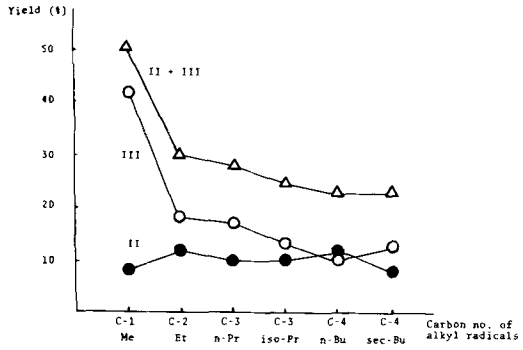

FIG. 2. The yields of radical alkylation products on α-tocopherol.

RESULTS AND DISCUSSION

The two kinds of main products were always derived from α-tocopherol, namely its alkyl ethers (IIx) and the cyclohexadienones (IIIx). Their structures were confirmed by the spectral data shown in Tables I and II. The mass spectra suggested that the two main products obtained from an alkyl radical have the same molecular weight and that each alkyl radical was attached to α-tocopherol with the abstraction of a hydrogen atom (i.e., M^+ 458 for ethyl, M^+ 472 for n- and iso-propyl, M^+ 486 for n- and sec-butyl).

The compound IIx species (IIb-f) were characterized as alkyl ethers of α-tocopherol; UV: λ_{max}^{hexane} 280-291 nm (aromatic), IR: ν_{max}^{neat} 1010-1020 cm^{-1} (C-O-C), ^{1}H-NMR: $\delta_{TMS}^{CDCl_3}$ 3.58-3.98 ppm (-O-CH_n-C-), ^{13}C-NMR: $\delta_{TMS}^{CDCl_3}$ 19.7-40.1 (isoprenyl C), 68.4-79.3 (-O-C-C-), 117.3-148.5 ppm (aromatic C). Further, the compound IIIx species were identified as cyclohexadienones by comparison with the spectra of IIIa (4), UV: λ_{max}^{hexane} 336-338 nm (dienone); IR: ν_{max}^{neat} 1630-1635 cm^{-1} (C=O), ^{1}H-NMR: $\delta_{TMS}^{CDCl_3}$ 1.83-2.01 ppm (C=C-CH_3), ^{13}C-NMR: $\delta_{TMS}^{CDCl_3}$ 11.1-14.3 (-C=C-CH_3), 19.7-40.2 (isoprenyl C), 116.3-147.5 (dien C), 204.1-204.5 ppm (C=O). The radical alkylation of α-tocopherol is depicted in Figure 1.

As shown in Figure 2, the yields of the cyclohexadienones (IIIx) were decreased with an increase of carbon numbers of alkyl radicals employed, but those of the alkyl ethers (IIx) did not vary greatly. It is noticed that methyl radical was highly effective in affording the cyclohexadienone type compound (IIIa). With sec-butyl radical, the yields of IIf and IIIf are 7 and 17%, respectively. However, these might not be comparable with the yields of the products from the other radicals, because the reaction with sec-butyl radical was carried out at higher temperature (110 C). These data suggest that it is relatively difficult for a bulky radical to attack the aromatic ring of α-tocopherol because of steric hindrance.

Generally, alkyl radicals appear to be scavenged by α-tocopherol. On the basis of the facts that the chromanoxy radical of α-tocopherol has been observed by electron spin resonance (ESR) (6) and that the alkyl ethers of it are obtained in all radical alkylations examined here, these alkylations are presumably initiated by the formation of chromanoxy radicals, which would be stabilized by radical delocalization. It is very interesting that the C-5 position is attacked specifically by alkyl radicals.

^{13}C-NMR data of the compound IIx and IIIx will be reported in detail on another occasion. We are now engaged in researches on the radical scavenging reactivity of α-tocopherol against other radicals.

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Effect of Dietary Vitamin E on Expiration of Pentane and Ethane by the Rat

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ABSTRACT

An analytical method for the measurement of hydrocarbon gases in the breath of rats is described. The method was used to follow the expiration in rat breath of in vivo formed scission products of hydroperoxides. The major products are pentane from the linoleic acid family and ethane from the linolenic acid family. Rats were fed 0, 11 or 40 i.u. vitamin E acetate/kg diet for 7 wk starting at age 21 days. Data obtained by gas chromatographic analysis of breath samples were analyzed by the Mann-Whitney nonparametric *U*-test. This statistical analysis showed that pentane evolved by the group of rats not supplemented with vitamin E was significantly higher during the period 1-7 wk than that evolved by either of the two supplemented groups of rats. Ethane from the nonsupplemented group was significantly higher than that from the group supplemented with 40 i.u. vitamin E/kg of diet by 5 wk, and significantly higher than both supplemented groups by 6 wk. By 7 wk, pentane production was tenfold greater in the nonsupplemented group than in either supplemented group, and ethane was about twofold greater. There was no significant difference between the groups supplemented with 11 and 40 i.u. vitamin E/kg diet for either ethane or pentane. This new technique, which measures scission products from in vivo lipid peroxidation, promises to be useful for application to many experimental areas where lipid peroxidation is expected or known to occur.

INTRODUCTION

There are many reviews of the scientific evidence showing vitamin E to function as a lipid antioxidant (1-6). Some of the evidence is shown by higher levels of malonaldehyde in tissue of rats not supplemented with vitamin E than in tissue of supplemented rats (7); by the presence of fluorescent products of lipid peroxidation that arise in part by malonaldehyde reaction with other biological compounds (8); by muscle degeneration and dystrophy (9); and by red blood cell fragility in vitamin E-insufficient animals as measured by hemolysis in vitro (9). Plaa and Witschi (10) concluded in a recent review that the concept of lipid peroxidation is one of the important concepts of current experimental pathology and toxicology, in spite of the fact that some of the most convincing evidence for the role of lipid peroxidation in vivo is indirect and that many conclusions have been drawn by inference. Recently, Riely et al. (11) showed that ethane production was characteristic of spontaneously peroxidizing mouse tissue in vitro and that carbon tetrachloride provoked formation of ethane in vivo. Abstracts by Hafeman and Hoekstra (12,13) describe the measurement of ethane to show protection by vitamin E and selenium against lipid peroxidation in vivo. Ethane is known to arise from autoxidizing linolenic acid (14), and it is a major thermolysis product of autoxidized linolenic acid (15).

Based upon the report by Riely et al. (11), we projected that not only ethane but also the linoleic acid hydroperoxide product pentane should be a useful index of lipid peroxidation in vivo. This paper describes a study of the effect of dietary vitamin E on the production of the hydrocarbon gases ethane and pentane as measured in breath samples from rats by a new analytical technique.

MATERIALS AND METHODS

Animals and Diets

Specific pathogen-free male rats, descendants of the Sprague-Dawley strain, were obtained at 21 days of age from Hilltop Lab Animals, Inc., Scottdale, PA. The rats were housed two per cage in filter-top plastic hanging cages for the duration of the study. The animals were kept on a 12-hr light and 12-hr dark cycle.

The basal diet used was that described by Draper et al. (16). Essentially, the diet contained in percentage: casein, 20; dextrose, 65.9; vitamin E-stripped corn oil, 10; and mineral and vitamin mix, 4. This basal vitamin E-deficient diet was fed to eight rats; vitamin E acetate was added to the basal diet at 11 i.u./kg and at 40 i.u./kg, and these supplemented diets were fed to groups of six rats each. The powdered

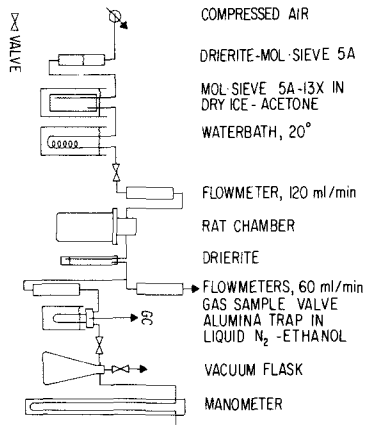

FIG. 1. Schematic diagram of breath sampling system.

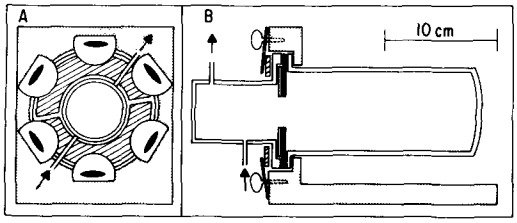

FIG. 2. Diagram of rat chamber drawn to scale. Shaded area is a plastic spacer placed between the wing nuts and head chamber; solid area is the rubber collar placed around the rat's neck; and stippled area is plastic holder for the chamber. (A) Front view; and (B) side view.

diet and tap water were given ad libitum. Food was removed from the cages 18-22 hr before breath samples were collected.

Gas Chromatography

A Varian-Aerograph model 1520 gas chromatograph with a flame ionization detector and fitted with a six-way gas sample valve was used for chromatographic analysis of hydrocarbon gases. A stainless steel column (1/8 in. X 5 ft) filled with activated alumina (80-100 mesh) was used with a nitrogen carrier gas flow rate of 25 ml/min. The detector temperature was 265 C and the injector temperature was 165 C. The column temperature was programmed as follows: 50 C for 1 min, followed by a 20 C rise/min for 7 min to a temperature of 190 C, which was held for 3 min and which was followed by a 20 C rise/min to 250 C. The total program was for 16 min. Between sample applications and after each day's analyses, the column and the alumina trap, to be described below, were held at 250 C with a nitrogen flow of 25 ml/min to remove any high molecular weight hydrocarbons.

Removal of Hydrocarbons from Air and Description of Animal Chamber

Figure 1 shows schematically the apparatus used to produce hydrocarbon scrubbed air and to collect the samples for injection into the gas chromatograph. Compressed air, at a tank pressure of 30 psi, was passed through a gas purifier (120 cc capacity) that was filled with indicating Drierite and molecular sieve 5A. Using 1/8 in. nylon or Teflon tubing and Swagelok fittings for all connections, the air was then passed through another gas purifier (stainless steel, 3.4 X 23 cm) that contained a mixture of molecular sieve 5A and 13X and that was immersed in a dry ice-acetone bath. After leaving the trap, the air passed through a coil of stainless steel tubing immersed in water at 20 C. The air then passed at a flow rate of 120 ml/min through a flowmeter, controlled by a valve, and into the stainless steel head portion of the animal chamber. The air stream, or air-breath stream when an animal was in the chamber, passed on the effluent side of the chamber through a tube that contained indicating Drierite (ca. 5 g) to remove water vapor, and then the air stream was split to obtain a flow rate of 60 ml/min through each of two flowmeters.

The body portion of the animal chamber, shown in Figure 2, was constructed of a glass cylinder that had a ground flanged opening. A rubber gasket with the appropriate size hole for the neck of the animal being tested was placed over the rat's head, followed by a rigid Teflon collar about 1.2 cm smaller in diameter and split on one side to allow its positioning around the rat's neck. It was determined that these two collars prevented the passage of any fecal gases, intestinal gases, or gases from the rest of the rat's body into the head chamber. The animal was placed in the glass chamber, and the stainless steel head chamber, with a glass viewing plate in the end, was placed over the head and held tightly against the rubber collar and the glass chamber flange with wing nuts screwed into a plastic holder. Two semicircular plastic spacers were placed between the head chamber and the wing nuts to apply sufficient pressure to allow complete sealing of the two chambers.

Collection of Breath Samples and Their Injection into the Gas Chromatograph

A length of nylon tubing was attached with Swagelok fittings to the inlet port of a six-way gas sample valve, mounted on the side of the gas chromatograph, and to the end of one of the two flowmeters on the effluent side of the rat chamber. The other flowmeter was always open to prevent a buildup of pressure in the chamber. Attached to the six-way gas sample valve in place of a standard gas sample loop was a loop of 1/8 in. stainless steel tubing, about 8 in. long, that contained activated alumina (80-100 mesh) in the lower 3 in. of the loop. During sample collection, this alumina trap was immersed in a very thick, icy slush of ethanol-liquid nitrogen. The trap was held immersed in the icy slush until the sample was injected into the gas chromatograph. The outlet port of the sample valve was attached to a 1-liter vacuum flask that had been evacuated with a small vacuum pump. The flow of air and breath into the alumina trap was controlled by opening a needle valve placed between the vacuum flask and the outlet side of the gas sample valve. The vacuum flask was attached to a calibrated mercury manometer. With a flow rate of 60 ml/min through the flowmeter and through the attached sample loop, 5 min were required to collect a 300-ml sample. Slight variations in flow rate through the flowmeter and through the attached sample loop were not a problem as 300-ml samples were always collected. After collection of the sample, the ethanol-liquid nitrogen bath was removed from around the sample loop trap, the nitrogen flow to the gas chromatograph was diverted through the trap via the sample valve, and a bath of hot tap water (70 C) was placed around the trap for the 3-min injection period. After 3 min, the nitrogen flow was again diverted from the sample loop trap directly into the chromatographic column.

Starting at 1 wk after initiation of the dietary regimen, each animal was placed in the holding chamber, and after 30 min, a 300-ml sample was collected. This sample represented one-half of the total breath sample in a 5-min interval. After chromatography, the relative peak areas of pentane and ethane were calculated by triangulation, and the picomoles of each gas in the 300-ml breath samples were calculated from standards injected via a 1 cc gas-tight syringe directly into the gas chromatograph. The standards, 0.8 ppm pentane and 1.5 ppm ethane in nitrogen, were obtained from Matheson Gas Products, Newark, CA. One milliliter portions of these standards were measured at an electrometer setting of 2 X 10^{-12} amps/mV. A 30-min time interval before collection of the breath sample was chosen to allow all the room air to be flushed from the tubing and from the head chamber and to accustom the rat to confinement in the chamber. After each day's use, the molecular sieve in the stainless steel trap used to clean hydrocarbons from the air was heated with a heating tape at about 350 C with a slow flow of nitrogen through the molecular sieve. Before animal breath samples were collected each day, a 300-ml sample of the background hydrocarbon-scrubbed air was collected and injected into the

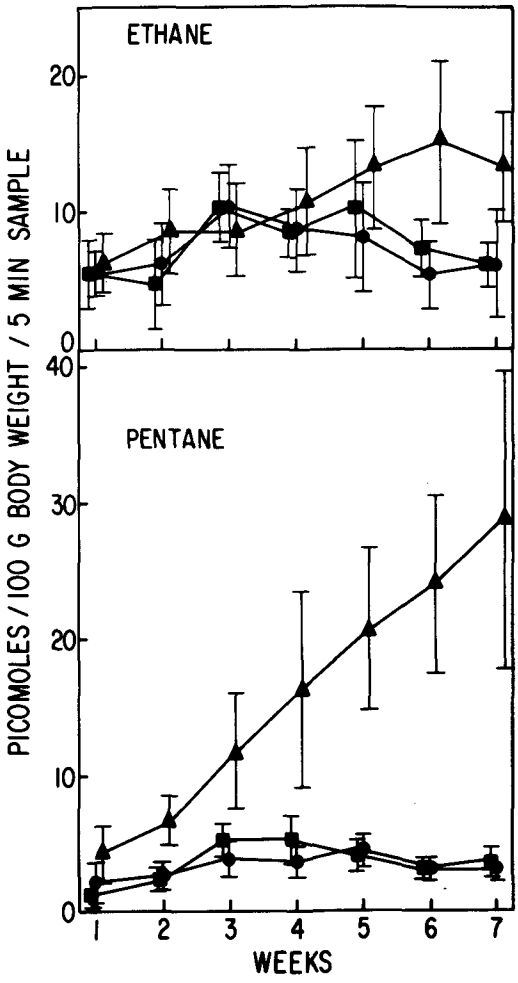

FIG. 3. (A) Ethane and (B) pentane/100 g body weight expired in one-half the total breath sample during a 5-min time interval beginning 30 min after placing a rat in the holding chamber. Diets fed for 7 wk beginning at 21 days of age contained (▲) 0, (■) 11, or (●) 40 i.u. vitamin E acetate/kg. The values plotted are the means ± S.D. for eight animals in the unsupplemented group and six animals in each of the vitamin E-supplemented groups.

TABLE I

Mann-Whitney *U*-Test Values[a] for Significant Differences Among Groups

Hydrocarbon gas	Groups compared[b]	Week of analysis[c]						
		1	2	3	4	5	6	7
Ethane	40 vs. 0	N.S.	N.S.	N.S.	N.S.	0.0294	0.0014	0.0046
	11 vs. 0	N.S.	0.042	N.S.	N.S.	N.S.	0.0046	0.0014
Pentane	40 vs. 0	0.0294	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006
	11 vs. 0	0.0026	0.0006	0.0006	0.0006	0.0006	0.0006	0.0006

[a]$\alpha(=2P) = 0.05$. Comparisons were made of picomoles ethane and pentane/100 g body wt. The sample of breath represented one-half the total expired air over a 5 min period.

[b]The groups were: six rats fed 40 i.u. vitamin E/kg diet; six rats fed 11 i.u. vitamin E/kg diet; and eight rats fed a vitamin E-deficient diet. There were no significant differences between the 40 and 11 groups for ethane or pentane.

[c]Rats were fed their respective diets beginning at 21 days of age and breath samples were analyzed weekly for the following 7 wk.

gas chromatograph to assure that the air the rats were exposed to was free of hydrocarbon gases, thus assuring that all the hydrocarbon gases measured were from the breath of the rat.

RESULTS

Among the hydrocarbon gases that chromatographed from the alumina column during the program described, those that were identified were methane, ethane, ethylene, propane, butane, pentane, and hexane. Of these compounds, only ethane and especially pentane increased dramatically in breath samples in rats fed a vitamin E-deficient diet over a 7-wk period.

The mean values ± S.D. for picomoles of pentane and ethane/100 g body weight are shown in Figure 3. These values represent one-half of the total expired air over a 5-min time interval beginning 30 min after a rat was placed in the holding chamber. The greater than tenfold difference between pentane expired and twofold difference between ethane expired by vitamin E-supplemented rats and the nonsupplemented rats by 7 wk of feeding the diets is striking. There was individual animal variation in pentane and ethane expired, as shown by the standard deviations; however, the α values in Table I for data shown in Figure 3, where $\alpha = 2P$, obtained by the Mann-Whitney *U*-test (17,18), a nonparametric statistical test, show the significant effect that the absence of vitamin E in the diet had on increasing pentane and ethane in the expired breath. The variations in amount of pentane and ethane shown by the standard deviations in Figure 3 are variations among the animals and are not variations in the analytical system. Gas standards collected in the same manner, but with one of the flowmeters on the effluent side of the chamber closed, showed that 100% of the injected standards could be collected consistently. The individual animal variations are not unlike those seen for measurements of hemolysis of red blood cells as a function of dietary vitamin E (9) and the scatter seen for muscle fatty acids as a function of dietary vitamin E (4). Pentane was significantly higher ($2P<0.05$) in the nonsupplemented rats as early as 1 wk after the rats were fed their respective diets. Ethane evolution was significantly higher by the nonsupplemented rats after 5 wk than by the rats supplemented with 40 i.u. vitamin E/kg diet, and by 6 wk it was significantly higher than that of both supplemented groups. There was no significant difference in the amounts of either ethane or pentane between the two groups of rats consuming 40 and 11 i.u. vitamin E/kg diet.

DISCUSSION

This investigation of the use of the hydrocarbon gases pentane and ethane as indices of lipid peroxidation was instigated by the report of Riely et al. (11) that ethane production in vivo was related to treatment of mice with carbon tetrachloride, a rapid inducer of lipid peroxidation in the liver (19). Hafeman and Hoekstra (12,13) reported the measurement of ethane to show protection by vitamin E and selenium against lipid peroxidation in vivo.

A search of the literature for references to the measurement of hydrocarbon gases shows that many volatile compounds have been measured from peroxidizing systems, especially food systems. Pentane evolved from peroxides during their decomposition has been correlated with sensory tests for rancidity (20,21), and flavor studies on pentane formation in foods have shown that it is possible to correlate linoleate content and iodine values with the

induction period for pentane formation (22). Pentane has been measured as a decomposition product of 13-hydroperoxyoctadeca-9,11-dienoic acid, a lipoxidase oxidized product from linoleic acid (15,23,24). In dehydrated food systems, oxidation processes were measured by thermal release of hydrocarbons and the predicted hydrocarbons were found: ethane from linolenic acid; octane from oleic acid; pentane from linoleic acid; pentene and butane from myristoleic acid, and heptene and hexane from vaccenic acid (25). Ethane was shown by Lieberman and Mapson (14) to be produced in nonenzymatic model systems of linolenic acid hydroperoxide where the decomposition was catalyzed by iron, and that the same reaction carried out with a cupric-ascorbate system yielded mainly ethylene. Lieberman and Hochstein (26) also reported enzyme-catalyzed generation of ethylene in rat liver microsomes in the presence of cuprous ions. Horvat et al. (27) also observed hydrocarbon gases during the oxidation of methyl linoleate, and Salke et al. (28) showed that saturated hydrocarbons arose early during autoxidation of soybean oil. Our own unpublished studies have confirmed the iron-catalyzed decomposition of purified linolenic acid and linoleic acid hydroperoxide with the release of ethane and pentane, respectively.

Analytical methods to detect lipid peroxidation in vivo have been quite limited. Other than measurements of red cell hemolysis (9) as a test to establish the state of vitamin E nutrition, there has been no method described to follow the course of vitamin E insufficiency, especially during the early stages of dietary experiments or pro-oxidant stress. This paper has described a technique that offers promise for application to many situations where lipid peroxidation is thought to occur in vivo. This technique is based upon the gas solid chromatography of hydrocarbons on activated alumina. List et al. (29) showed that the technique readily separates C_1 to C_8 paraffins from α-olefins, and that more polar materials such as aldehydes, ketones, and esters are irreversibly adsorbed by the alumina.

The finding that pentane in the breath of rats fed a diet that contained vitamin E-stripped corn oil was significantly higher than in the breath of vitamin E-supplemented rats after only 1 wk is not surprising if one considers that corn oil contains 56-57.5% linoleic acid (9,30). The linoleic acid would be rapidly incorporated into membranes of the growing rat. For example, the total lipids of liver mitochondria from rats that consumed 160 mg of linoleic acid per day from a standard rat diet contained 23.4% linoleic acid, 17% arachidonic acid, and 6.2% docosahexaenoic acid (31). Vitamin E insufficiency would lead to the peroxidation of membrane lipids with the formation of hydroperoxides of the linoleic family, the ω-6-unsaturated fatty acids. In a similar manner, linolenic acid and docosahexaenoic acid, both ω-3-unsaturated fatty acids, would peroxidize to yield ethane as one product. Organic hydroperoxides are known to greatly increase the effectiveness of hemoproteins, cytochromes and hematin in catalyzing lipid peroxidation (32). The order of effectiveness in catalyzing lipid peroxidation was shown to be similar to their order of effectiveness in decomposing hydroperoxides (33). Heme compounds could catalyze the decomposition of hydroperoxides of the linoleic family in vivo to yield among other products, low levels of pentane.

The general route for formation of pentane and ethane from free radical products of hydroperoxide decomposition can be readily visualized. Considering the route to the formation of pentane, some of the ω-6-hydroperoxides can be decomposed by iron:

$$CH_3(CH_2)_4\underset{OOH}{C}\text{-R} + Fe^{++} \rightarrow$$

$$CH_3(CH_2)_4\underset{O\cdot}{C}\text{-R} + Fe^{+++} + OH^-$$

This alkoxy radical would readily undergo β-scission (34) with the production of $CH_3(CH_2)_3\dot{C}H_2$. This pentane radical would form pentane by hydrogen abstraction.

The protection afforded by dietary vitamin E, as shown by lower levels of ethane and pentane in vitamin E-supplemented rats, could be mediated by its chain breaking antioxidant action. The kinetics and mechanisms of chain scission during peroxidation and from hydroperoxide decomposition to yield hydrocarbon gases in vivo are not known. However, studies are underway to determine in vitro decomposition of purified linolenic and linoleic acid hydroperoxides to yield ethane and pentane, respectively.

ACKNOWLEDGMENTS

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The Phytanic Acid Content of the Lipids of Bovine Tissues and Milk

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ABSTRACT

In three steers which were given grass silage for six months, the content of phytanic acid (i.e. 3,7,11,15-tetramethylhexadecanoic acid) in plasma lipid increased to about 8% of the total fatty acids, whereas after this time the proportion in the total fatty acids of liver and heart lipids was about 1%, and only 0.1% in those of kidney lipids; the acid was present in trace amounts in adipose-tissue triglycerides and was apparently absent from brain lipids. In eight lactating cows which were given grass silage for about 3 months, the content of phytanic acid in the total long chain fatty acids of milk and of plasma was 0.7% and 13%, respectively. In the plasma lipids of both steers and lactating cows, phytanic acid constituted a substantial proportion of the total fatty acids of the triglycerides and phospholipids; the acid was present in lowest proportion in the cholesteryl esters.

INTRODUCTION

The multibranched fatty acid, phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) is a normal constitutent of the lipids of the plasma, tissues, and milk of ruminant animals (1). The carbon skeleton of the acid is of exogenous origin and in herbivores is apparently largely derived from phytol, the alcohol moiety of chlorophyll (1,2). In cattle, the proportion of phytanic acid in the total fatty acids of plasma lipids was found to vary widely depending on the composition of the feed ingested (1). Phytanate was present in abundance in the plasma lipids of cows given grass silage (1) and comprised up to 40% of the fatty acids of plasma phospholipids isolated from a cow which had been given ensiled grass (3).

In rats, mice, rabbits, and chinchillas given experimental diets that were rich in phytol or phytanic acid, the multibranched acid was found to accumulate, not only in the plasma lipids, but also in tissue lipids, notably those of kidney, liver, and heart (4-6). It was therefore decided to investigate the lipids of the plasma, tissues, and milk of cattle in relation to the accumulation of phytanate which can be induced when they are fed on grass silage; some observations were also made on phytanic acid in the lipids of maternal and foetal plasma.

METHODS AND EXPERIMENTAL PROCEDURES

Lipid Extraction

Plasma samples were freeze-dried, and water amounting to one-fifth of the original volume of plasma was added to the residue. The partially reconstituted plasma was extracted with chloroform-methanol (2:1 v/v) as described by Folch et al. (7). Tissue samples which had been freshly excised or which had been stored at -20 C were also extracted by the method of Folch et al. (7) as were the samples of milk lipid that were obtained by centrifugation of freshly-collected milk.

Lipid Fractionation

Lipids were separated by silicic acid chromatography into fractions comprising cholesteryl esters, triglycerides, and phospholipids; unesterified fatty acids were isolated by treatment of the neutral lipids with aqueous KOH according to Garton et al. (8). Methyl esters from each lipid fraction were prepared as previously described (9).

Gas Chromatography

The analysis of methyl esters of long chain fatty acids was made using Apiezon L and ethyleneglycol-adipate polyester (EGA) as stationary phases (9). Since methyl phytanate is superimposed upon heptadecanoate when eluted from EGA and by octadecadienoate and octadecatrienoate on the nonpolar phase, determination of the multibranched acid was made on Apiezon L after removal of the unsaturated fatty acid esters using mercuric acetate or by their prior conversion to saturated esters by catalytic hydrogenation (1).

Experiment A

Three Friesian steers which from weaning had been reared on barley supplemented with hay and dried grass, were given grass silage only for a period of about 6 months, after which they were killed. Blood samples were taken at the start of silage-feeding and on three subsequent occasions; the final sample was taken 3

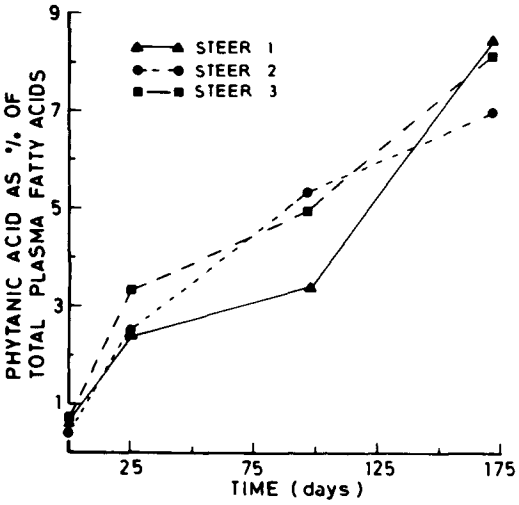

FIG. 1. Changes in the proportion of phytanic acid in the total fatty acids of the plasma lipids of three steers given grass silage to appetite.

days before slaughter. Tissue samples were taken immediately after slaughter and were stored at -20 C for not longer than 4 wk before they were analyzed.

Experiment B

Samples of milk and of plasma were taken from eight Friesian cattle which were in their first lactation and which had been given grass silage only for about three months. Three of the eight samples of plasma lipids were fractionated; five samples were examined without prior fractionation.

Experiment C

Two pregnant Friesian heifers were given grass silage supplemented with concentrates comprised of barley, oats, and linseed meal. After parturition, blood samples were taken from the calves before they were suckled. Blood samples from the heifers were taken at calving.

The animals used in Experiments A and C were from the Duthie Experimental Farm, Rowett Research Institute, Aberdeen, while those used in Experiment B were from Grange Farm, County Meath, The Agricultural Institute, Ireland.

RESULTS

The changes in the content of phytanate in the plasma lipids of the three animals in Experiment A are shown in Figure 1 which indicates that, within 4 wk of the commencement of silage feeding, the proportion of the multibranched acid had increased markedly. The phytanate content of plasma lipids continued to increase throughout the experiment and was apparently still increasing at the time the final blood sample was taken. At slaughter the values for the proportion of phytanic acid in the total fatty acids of plasma were 6.9, 8.2, and 8.4%.

The composition of the fatty acids of the main lipid classes and the contribution which the fatty acids from each class made to the plasma total fatty acids of the plasma samples obtained 97 and 171 days after the commencement of silage feeding are shown in Table I.

In agreement with previous findings on bovine plasma (3), phytanic acid was distributed throughout the main lipid classes. In the present investigation, the proportions of phytanate which contributed to the total fatty acids of the triglycerides and of the phospholipids, respectively, were similar, though in two of the blood samples which were taken at 97 days, the proportion of phytanate in glyceride fatty acids exceeded that in the phospholipid fatty acids. The proportion of phytanic acid in the unesterified fatty acids was considerably lower than in the glyceridic lipids, while only traces were found in the cholesteryl ester fraction. It should be noted that, with time, the proportion of phytanic acid present in any given lipid class may change depending on the turnover time of the phytanyl residues in relation to their site of esterification within glycerolipid molecules. Given a fairly constant intake of phytol, it is possible that an "equilibrium state," with respect to lipid classes in a particular organ, might be reached, but this was probably not the case in the present experiment in which continuously increasing levels of phytanate were observed in plasma total lipids.

The proportions of phytanate in the component fatty acids of tissue lipids are given in Table II, which shows that phytanate was present as a minor constituent in the lipids of liver, kidney, and heart. Table II also shows that phytanate was only a trace component of the fatty acids of omental depot lipids, and was apparently absent from the lipids of brain. For the most part, the phytanate content of the fatty acids of the main lipid classes of the tissues was less than 1%; only the triglycerides of liver contained appreciable amounts (up to 8%).

The mean values for the proportion of phytanate in the total fatty acids of the lipids of liver and heart were 1.2% and 1.1% respectively, so that about a sevenfold difference existed between these values and the corresponding proportion (7.9%) of phytanate in the plasma total fatty acids.

TABLE I

Content of Phytanic Acid in the Main Lipid Classes of Plasma from Three Steers Given Silage[a]

Days in silage	Steer 1		Steer 2		Steer 3	
	97	171	97	171	97	171
Cholesteryl esters	tr[b] (42.9)	tr (41.6)	tr (37.9)	tr (39.3)	tr (43.4)	tr (38.4)
Triglyceride	11.5 (3.3)	14.4 (7.0)	9.1 (6.7)	8.4 (6.7)	13.7 ((4.9)	8.9 (8.1)
Unesterified fatty acid	2.1 (5.2)	2.9 (1.7)	1.8 (4.9)	0.7 (3.4)	0.9 (13.5)	2.5 (2.5)
Phospholipid	6.0 (48.6)	14.8 (49.7)	9.1 (50.5)	12.5 (50.6)	10.7 (38.2)	14.5 (51.0)

[a]Values expressed as percengage of total fatty acids; the percentage contribution of the fatty acids of each lipid class to the total fatty acids, is given in parentheses.

[b]tr = trace amount (i.e. <0.2%)

TABLE II

Content of Phytanic Acid in the Main Lipid Classes of Liver, Kidney and Heart, and in the Total Lipids of Brain and of Omental Depot Tissues, of Three Steers Given Grass Silage for 174 days[a]

Tissue	Lipid	Steer 1	Steer 2	Steer 3
Liver	Cholesteryl ester	ND[b] (2.8)	1.7 (2.1)	ND (4.8)
	Triglyceride	7.9 (2.6)	3.7 (9.5)	2.2 (14.3)
	Unesterified fatty acid	0.4 (11.4)	0.7 (4.3)	0.5 (1.8)
	Phospholipid	0.9 (83.2)	1.8 (84.1)	0.4 (79.1)
Kidney	Cholesteryl ester	tr[c] (4.2)	tr (3.4)	ND (4.6)
	Trilgyceride	0.4 (6.7)	0.4 (1.9)	0.3 (1.7)
	Unesterified fatty acid	ND (10.8)	ND (24.9)	tr (17.4)
	Phospholipid	0.3 (78.3)	ND (69.2)	ND (76.3)
Heart	Cholesteryl ester	tr (6.1)	1.0 (4.7)	1.9 (5.8)
	Triglyceride	2.1 (16.4)	0.7 (26.3)	2.7 (33.4)
	Unesterified fatty acid	tr (8.6)	tr (5.5)	0.4 (5.8)
	Phospholipid	0.4 (68.9)	0.5 (63.5)	2.3 (55.0)
Brain	Total	ND	ND	ND
Omental depot	Total	ND	tr	tr

[a]Values expressed as percentage of phytanate in total fatty acids in each lipid class, or in total lipid, the percentage contribution of the fatty acids of each lipid class to the total fatty acids, is given in parentheses.

[b]ND = not detected

[c]tr = <0.2%

TABLE III

Content of Phytanic Acid in the Total Long Chain Fatty Acids of Plasma and of Milk of Eight Lactating Cows Given Grass Silage; the Proportion of Phytanate Contributing to the Total Fatty Acids of the Main Lipid Classes of the Plasma Lipids of Three of the Eight Animals[a]

	Plasma lipids					Milk lipids
	Cholesteryl ester	Triglyceride	Unesterified fatty acid	Phospholipid	Total	Total
Cow	1.1 (35.2)	15.2 (8.4)	7.6 (1.0)	23.9 (55.4)	15.0	1.0
Cow	0.9 (39.3)	18.0 (4.5)	8.3 (1.3)	20.9 (54.9)	12.7	0.5
Cow	0.6 (40.8)	17.7 (5.3)	8.1 (1.3)	22.1 (52.6)	12.9	0.8
Mean of 8 cows					13.2 ± 1.4	0.69 ± 0.19

[a]Values expressed as percentage of total fatty acids; the percentage contribution of the fatty acids of each lipid class to the total fatty acids is given in parentheses.

The results of the analysis of blood-plasma lipids and of milk lipids obtained in Experiment B are shown in Table III; the values respresenting the proportions of phytanate in milk lipids are expressed in terms of total long chain fatty acids (i.e. acids of chain-length $>C_{12}$) and are

therefore about one-third greater than might be expected from calculations based on the entire complement of fatty acids of these lipids (10).

From Table III, it can be seen that, whereas phytanic acid accounted for up to 15% of the total fatty acids of plasma, it was present only as a minor constituent of the fatty acids of milk. Seven of the eight samples of milk had a phytanate content that was less than 1% of the total long chain fatty acids. Comparing the mean values shown in Table III, the extent of the disparity in the proportions of phytanate in the fatty acids of plasma and of milk is about twentyfold. A similar order of difference also exists between the proportion of phytanate in the fatty acids of the plasma triglycerides and that in the long chain fatty acids of milk fat.

With regard to the content of phytanate in the main lipid fractions of plasma (Table III), it can be seen that the pattern of distribution of the multibranched acid is closely similar to that observed in the experiment in which steers were used (Table II). Thus, in the sample of plasma taken after 171 days on experiment, the phospholipid fraction contained the major proportion of the total plasma phytanate. Furthermore, the contribution of phytanate to the total fatty acids of each lipid fraction was greatest in the phospholipid class.

Results obtained in Experiment C showed that, whereas the proportion of phytanic acid in the total plasma lipids of maternal plasma were 5.6% and 7.4% phytanic acid was not detected in the total fatty acids of calf plasma.

DISCUSSION

The finding of phytanate in high proportions in the plasma lipids of cows given grass silage confirms a previous observation (3). In addition, it was found that the multibranched acid was present in greatest proportion in the fatty acids of phospholipids and occurred in smallest proportion in the fatty acids of the cholesteryl esters, thus exhibiting a pattern of distribution between the lipid classes which was similar to that reported in earlier work (3).

In the experiment using steers, the presence over some months of phytanate in substantial proportion in the triglycerides and phospholipids of plasma was not associated with its deposition in quantity in any of the tissues studied. The virtual absence of phytanic acid from brain lipids is especially noteworthy because these lipids have been the subject of considerable interest in connection with the possible relationship between the occurrence of the acid in nervous tissue lipids and the neuropathology of Refsum's Syndrome in man (1).

The lack of phytanate in the adipose tissue lipids of the steers is perhaps not surprising since experiments which involved the feeding of phytol, dihydrophytol, or phytanic acid to rats resulted in the assimilation of only very small amounts of phytanate into depot lipids, while marked accumulation of the acid in the lipids of kidney, liver, and heart occurred (4,5). Because there is considerable evidence to suggest that triglycerides are hydrolyzed to unesterified fatty acids during their uptake from plasma into adipose tissue (11,12), it is probably of relevance that the activity of lipoprotein lipase towards glycerides containing phytanyl groups is considerably lower than that towards glycerides composed of normal acids. Thus, Laurell (13) observed that lipoprotein lipase was inactive towards glyceryl triphytanate, whereas the enzyme readily hydrolyzed glyceryl tripalmitate. Later, Ellingboe and Steinberg (14) confirmed Laurell's findings and further showed that there was virtually no hydrolysis of glyceryl 1,3-diphytanyl-2-palmitate, but that there was significant hydrolysis of the phytanyl ester bond of triglycerides containing one phytanyl group in either the 1- or 2-position. While it would seem unlikely that triglycerides containing two or three phytanyl groups were present to any extent in the plasma lipids of the cows and steers used in the present experiments, there would appear to be little doubt that, as previously suggested (13,14), the low proportions of phytanic acid in the depot lipids are to some degree the result of the inhibitory action of the branched chain structure of the acid towards lipoprotein lipase.

The low activity of lipoprotein lipase towards phytanyl ester bonds of glycerides may also be of significance in relation to the observation that the phytanate content of milk lipid is low compared with that of plasma triglycerides. It is known that triglycerides are taken up from plasma by the cow mammary gland and that fatty acids which are released from ester combination enter a metabolic pool before they are incorporated into milk lipids (15). Since almost the entire complement of C_{18} fatty acids in cow milk fat is derived from plasma glycerides (15), the marked difference in the proportion of phytanic acid between plasma glycerides and milk lipids would suggest that there exists a high degree of selectivity against the incorporation of phytanic acid into milk lipids. While it is likely that this selectivity is due to the relative inactivity of hydrolytic enzymes towards phytanyl ester groups, other

conditions may also be contributory. Thus, in their consideration of factors influencing the deposition of phytanate in adipose tissue, Ellingboe and Steinberg (14) suggested that the kinetics of enzymes catalyzing the esterification and transesterification of phytanic acid might be affected by the branched chain structure of the acid. Thus, while the enzyme system cholesterol:lecithin acyltransferase functions in bovine plasma (16), the proportion of phytanate in the form of cholesteryl ester is very small in relation to that present in phospholipids. Whether the lack of cholesteryl phytanate is due to the inactivity of the transferase towards the branched chain acid or to the absence of the phytanyl group from the 2-position of lecithin is not known.

It was observed that the proportion of phytanate in the total fatty acids of the plasma of lactating cows given silage was greater than in that of silage-fed steers. While the experimental conditions used in the two experiments differed, thus precluding a direct comparison, it is apparent from the results reported by Duncan and Garton (3) that lactation is associated with an increase in the proportion of phytanate in the plasma triglycerides and phospholipids of cows receiving grass-silage. This may be due, in part, to increased feed consumption associated with the nutritional demands of lactation. However, it is likely that phytanate will accumulate in plasma lipids when the availability of phytanate to the mammary gland is limited because mammary-gland lipoprotein lipase discriminates against phytanyl groups present in glycerides (13).

The apparent absence of phytanic acid from the plasma total fatty acids of two new-born calves (17) suggests that, in cows, the placental barrier is virtually impermeable to the multibranched acid. In a study of tissue lipids of the new-born lamb, Downing (18) was unable to detect any multibranched fatty acids, though there was evidence to suggest that some placental transference of monomethyl-branched acids had occurred.

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Uptake and Metabolism of Fatty Acids by Soybean Suspension Cells

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ABSTRACT

Soybean suspension cultures very rapidly take up C_{16} and C_{18} fatty acids by a nonspecific, nonenzymic binding of exogeneously added fatty acids to cell walls and by a subsequent transfer into the cell where they are rapidly incorporated into triacylglycerols, phosphatidylcholines, and phosphatidylethanolamines. ^{14}C-Palmitic and ^{14}C-stearic acids follow this sequence but are not desaturated, wherease ^{14}C-oleic and ^{14}C-linoleic acids are transferred more rapidly than the saturated fatty acids and are then further modified. All the data fit a sequence of events by which free oleic acid is first activated to a CoA thioester, and then desaturated to linoleyl-CoA; both thioesters are then transferred to triacylglycerols, phosphatidylcholine, and phosphatidylethanolamine.

INTRODUCTION

In contrast to intact or sliced plant tissues, plant cell suspension cultures can serve as reproducible sources of homogeneous cells that can be readily grown and manipulated for biochemical studies. However, since these cells are grown in liquid media, they lack anatomical organization and their metabolism may thereby be altered by these unique conditions. Nevertheless, their use as model plant cell systems is being actively exploited for biochemical studies (1).

Until recently, plant cell suspension cultures have not been employed extensively for studies related to lipid metabolism. Current literature on this subject has been reviewed by Radwan and Mangold (2). Moore and Beevers (3) have recently described a number of parameters involving the use of soybean suspension cells. Stearns and Morton (4,5) have examined the capacity of soybean suspension cells to incorporate [1-^{14}C]acetate into fatty acids as well as the effect of growth hormones on lipid biosynthesis. They observed that within 6 hr, ^{14}C derived from [1-^{14}C]acetate was incorporated into the acyl moieties of phospholipids and was distributed among the saturated, mono-, di-, and trienoic fatty acids.

This paper will describe experiments designed to examine the capacity of soybean suspension cells to metabolize exogeneously added C_{16} and C_{18} fatty acids.

EXPERIMENTAL PROCEDURES

Soybean Suspension Cultures

Suspension cultures of Soybean (*Glycine max* L.) were grown on B5 medium containing 2 ppm 2,4-D (6). The cultures were incubated in the dark at 28 C with continual shaking and subcultured at intervals of 3-5 days. Cell cultures, grown for 5 days, were harvested by suction filtration, weighed, and immediately employed for experimental purposes. Preliminary experiments indicated that over a 1-10 day growth period, uptake and metabolic activity for fatty acids were quite constant.

Ghost Cells

Ghost cells ere prepared by suspending 12 g (wet weight) 5 day old suspension cells in 200 ml of a chloroform-methanol mixture (2:1 v/v) for 4 hr at room temperature with occasional shaking. The cells were then filtered off by suction, washed once with acetone and 3 times with diethylether, and dried under vacuum until the weight remained constant. Final weight of dried cells was 590 mg. Thus, 25 mg of these cells was equivalent to 0.5 g of fresh cells (wet weight).

Chemicals

[1-^{14}C]Acetate (58 mCi/mmol), [1-^{14}C]-palmitic acid (56 mCi/mmole), [1-^{14}C]stearic acid (58 mCi/mmole), [1-^{14}C]oleic acid (54 mCi/mmole), and [1-^{14}C]linoleic acid (61 mCi/mmole) were purchased from the Radiochemical Center, Amersham, England. All solvents employed were reagent grade and were also freshly distilled. Silica Gel G (type 60) was obtained from Merck (Darmstadt, Germany). For argentation Thin Layer Chromatography (TLC), 10% $AGNO_3$ Silica Gel G plates were prepared, activated, and stored in the dark before use. Lipase (*Rhizopus arrhizus*) and

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TABLE I

Incorporation of ^{14}C-Substrates into Fatty Acids by Soybean Suspension Cells

^{14}C-Substrates[a]	% Incorporation					
	16:0	18:0	18:1	18:2	18:3	20:0
Acetate	19	t	58	18	0	5
Palmitate	100	0	0	0	0	0
Stearate	0	100	0	0	0	0
Oleate	0	0	83	17	0	0
Linoleate	0	0	0	95	<5	0

[a]Each reaction mixture contained 2 g (wet weight) soybean suspension cells (5 day old), 150 μmoles of phosphate buffer (pH 7.3), and, where indicated, sodium [1-^{14}C]acetate, 45 nmoles (2.5 μCi); [1-^{14}C]palmitate, 45 nmoles (2.5 μCi); [1-^{14}C]stearate, 43 nmoles (2.5 μCi); [1-^{14}C]oleate, 46 nmoles (2.5 μCi); and [1-^{14}C]-linoleate, 44 nmoles (2.5 μCi) in a total volume of 1.51 ml. Reaction time 5 hr at 25 C in a 25 ml scintillation vial with vigorous shaking in a Braun-Warburg bath. At end of reaction time, ^{14}C products were analyzed as described in text.

Phospholipase A_2 (*Crotalus terr. terr.*) were purchased from Boehringer-Mannheim GmbH Mannheim, West Germany.

Incubation Conditions

The basic incubation system consisted of 0.5 g suspension cells (wet weight), 5 days old, 3 ml 0.1 M phosphate buffer [pH 7.3 and 10 μl of ^{14}C substrate (0.2 μCi/54 nmoles) dissolved in ethyleneglycolmonomethylether] in a scintillation vial of 25 ml capacity. The reaction system was shaken in a Braun-Warburg shaker at 25 C for a given period of time. At the appropriate time, the reaction mixture was decanted from the vial into a centrifuge tube and centrifuged 1 min at 1000 x g. Fifty μl of the clear supernatant were assayed for ^{14}C content and the pellet was further examined by either of the following two methods.

(a) Fatty acid analysis: 1 ml of 20% methanolic KOH was added to the entire pellet, the tube capped and heated to 90 C for 30 min, and the solution cooled, acidified, and extracted two times with 1 ml chloroform. The chloroform extract was evaportated to dryness at 40 C with a stream of N_2; the lipid residue was dissolved in an excess of a freshly prepared ethereal solution of diazomethane. After 20 min at 0 C, the still yellow solution was evaporated to dryness by a stream of N_2; the residue was dissolved in a minimum volume of benzene and suitable aliquots spotted on $AgNO_3$-TLC plates. The plates were developed at room temperature with a chloroform-ethanol-acetic acid solvent system (99:1:0.1 v/v/v). The radioactive areas of the readily separated methyl esters of saturated, mono-, di-, and trienoic acids were detected by a scanner system manufactured by Frieseke and Hoepfner, Erlange-Bruck, Germany.

(b) Complex lipid analysis: 1 ml of hot isopropanol was added to the pellet and the suspension was thoroughly mixed on a vortex mixer, reheated for 5 min at 100 C, and cooled, and then 5 ml of a chloroform-methanol mixture (2:1 v/v) was added, shaken, and allowed to stand for 1 hr. Then 1 ml of water was added, shaken on a vortex mixer, and centrifuged for 5 min to separate the two phases. Then the chloroform layer was carefully removed with a Pasteur pipette and evaporated to dryness at 40 C by a stream of N_2. The lipid residue was dissolved in a minimum volume of chloroform, suitable aliquots spotted on Silica Gel G TLC plates and developed with a chloroform-methanol-water system (65:25:3 v/v/v). Consistent Rf values obtained for triacylglycerols (TG), free fatty acids (FFA), phosphatidylethanolamines (PE), and phosphatidylcholines (PC) were 0.95, 0.85, 0.63, and 0.30, respectively. TLC plates were scanned for ^{14}C content as previously described.

Uptake Studies

Freshly harvested cells, 0.5 g (wet weight), were suspended with shaking on a vortex mixer in 3.0 ml of 0.1 M phosphate buffer (pH 7.3) in a vial of 25 ml capacity. At zero time, 10 μl of ^{14}C substrate (0.2 μCi/54 nmoles dissolved in ethyleneglycolmonomethylether) were added, the suspension thoroughly mixed and incubated with shaking at 25 C for the indicated time. At stated intervals, vials were rapidly decanted into centrifuge tubes and then centrifuged for 1 min in 1000 x g. Fifty μl of the clear supernatant were counted for ^{14}C content.

This procedure was developed after it was observed that suction filtration of a solution of ^{14}C fatty acids in phosphate buffer through a Whatman #1 filter paper disc resulted in high retention of ^{14}C fatty acids on the discs.

TABLE II

Aerobic Requirement for Desaturation[a]

Gas phase	nmoles per hr	
	18:1 → 18:2	18:2 → 18:3
N_2	0	0
Air	22	10
O_2	19	5

[a]Experimental conditions described in Experimental Procedures.

TABLE III

Uptake of ^{14}C Fatty Acids by Ghost Cells

Substrate[a]	% Uptake from media Time (min)				
	"0"	10	30	60	120
Palmitate	19	46	72	–	73
Stearate	13	40	60	76	–
Oleate	40	83	91	–	–
Linoleate	60	86	86	–	–

[a]Each reaction mixture consisted of 25 mg dried soybean suspension cells (see Ghost cell preparation in Experimental Procedures) which is equivalent to 0.5 g (wet weight) of cells: 300 μmoles of phosphate buffer (pH 7.2); ^{14}C-substrate, 0.2 μCi/54 nmoles in a total volume of 3 ml. See Experimental Procedure section for further details. "O" time is an operational term defined as the time from addition of substrate to suspension cells, vortex mixing, centrifugation for 1 min and immediate removal of 50 μl aliquot from supernatant. Approximately 30 sec elapsed before centrifugation was begun.

Identification of Products

^{14}C-Labelled fatty acids derived from [1-^{14}C]acetate incubations were analyzed first by converting the ^{14}C-acyl moieties into their methyl esters as described above and then by separating and detecting the ^{14}C-components with a Packard Model 419 Becker Gas Chromatograph connected to a Packard Model 894 Gas Proportional Counter. Identification of the ^{14}C-methyl esters was made by co-chromatography with authentic methyl esters of fatty acids.

Phospholipids were identified by a comparison of the behavior of ^{14}C products with authentic samples on Silica Gel G-TLC with chloroform-ethanol-water (65:25:3 v/v/v) and by association of the ^{14}C area with a positive molybdenum blue color after spraying with the Dittmer and Lester reagent (7). Finally, the ^{14}C area tentatively identified as PC was positively identified by giving a positive reaction with the Dragendorff reagent (8); the ^{14}C area assigned to PE gave a positive reaction with ninhydrin. ^{14}C-TG were identified by co-chromatography with authentic triacylglycerol both with the chloroform-ethanol-water (65:25:3 v/v/v) system and with the hexane-diethyl ether-acetic acid (90:20:0.5 v/v/v) system and by the reaction of the ^{14}C product with a fungal lipase (*Rhizopus arrhizus*).

The desaturation products of ^{14}C-oleic acid and ^{14}C-linoleic acid obtained by incubating the substrates with suspension cells, namely ^{14}C-linoleic and ^{14}C-linolenic acid, respectively, were identified (a) by argentation TLC of the methyl esters with chloroform-ethanol-acetic acid (95:1:0.1 v/v/v) as the solvent system, and (b) by co-chromatography of the ^{14}C products with authentic methyl linoleate and linolenate employing a Packard Model 419-894 GLC-radio monitoring unit as the detection system. The column was 2 meters long and 2.7 mm in diameter and consisted of a 5% EGSSX on Gaschrome Q80-100 mesh at 170 C.

Radioactivity was determined by counting aliquots in 10 ml of New England Nuclear Aquasol-2 cocktail with a Packard model 2425 Tri-Carb Liquid Scintillation Spectrometer System.

RESULTS AND DISCUSSION

Conversion of Substrates by Culture Suspensions

Stumpf and Porra (9) observed that slices of developing soybean cotyledons converted [1-^{14}C]acetate rapidly into ^{14}C-palmitate, ^{14}C-stearate, ^{14}C-oleate, ^{14}C-linoleate, and ^{14}C-linolenate but that [1-^{14}C]palmitate and [1-^{14}C]stearate were ineffective substrates for elongation and desaturation, whereas [1-^{14}C]-oleate was readily converted to [1-^{14}C]linoleate.

As summarized in Table I, we have obtained similar results with soybean suspension cultures, i.e, [1-^{14}C]acetate was rapidly incorporated in C_{16} and C_{18} fatty acids whereas [1-^{14}C]palmitate and [1-^{14}C]stearate were not modified. [1-^{14}C]Oleate was, however, rapidly converted to [1-^{14}C]linoleate and to a lesser extent, [1-^{14}C]linoleate to ^{14}C-linolenate. The desaturation reactions were dependent on aerobic conditions as summarized in Table II. These results can be readily explained in terms of an ACP dependent de novo sequence which converts acetate to palmityl-ACP, a specific elongation system which elongates palmityl-ACP to stearyl-ACP (10), and finally a high specific stearyl-ACP desaturase which converts stearyl-ACP to oleyl-ACP (11). Since free palmitic and stearic acids cannot be converted to acyl-ACP derivatives, these acids do not enter the acyl-ACP pool and hence are neither elongated nor desaturated. Subsequent

desaturations would involve oleyl-CoA as the substrate for the formation of linoleyl-CoA, etc. The overall relationship of ACP and CoA acyl thioesters in fatty acid biosynthesis and modifications is discussed by Shine et al. (12).

Uptake of Free Fatty Acids by Suspension Cultures

In order to understand more fully the capacity of suspension cultures to utilize exogeneously added free fatty acids (FFA), a series of experiments were designed to examine the kinetics of FFA uptake by these cells.

Since preliminary experiments showed a very rapid uptake of FFA by fresh cells, it was essential to determine if nonviable cells were equally able to take up FFA from the media. When ghost cells (see Experimental Procedures for description of their preparation) were employed, a similar rapid uptake was observed. As summarized in Table III, within 10 min over 40% of the exogeneously added saturated FFA became associated with nonspecific binding sites of the cell walls and over 80% of the unsaturated FFA were bound to the cell wall. Thus, it could be concluded that the initial phase of uptake of exogeneous FFA would include a physical, nonenzymic association of FFA with cell wall components of the ghost cell. All of the ^{14}C-label was associated as FFA.

Similar time studies with fresh cells showed a very rapid uptake of exogeneously added FFA with a rapid movement of the ^{14}C-label into TG, PC, and to a lesser extent PE. Figures 1, 2, and 3 summarize the data for the uptake of C_{18} FFA by suspension cultures.

The following observations are noteworthy:

(a) When [1-^{14}C]stearate is added to a suspension culture, within less than a minute (the operational "O" time as defined in Table III) 98% of the bound ^{14}C-label was FFA and only 2% was associated with PC. In sharp contrast, when either [1-^{14}C]-oleate or [1-^{14}C]linoleate was added, in the same time interval, 50% of the oleate and 33% of the linoleate were now associated with complex lipids.

(b) The most rapidly labeled complex lipid was consistently PC. Of interest, 90% of the ^{14}C-label was associated with the C_2 position of the glyceryl moiety. Both TG and PE were labeled but at slower rates.

(c) Equally interesting was the consistent observation that the desaturation of 18:1 to 18:2 and 18:2 to 18:3 occurred only after a lag of about 30 min despite the early presence of ^{14}C-labeled PC, a proposed substrate for the desaturation process in plants (13).

(d) Furthermore, substrate and products, namely 18:1 to 18:2 and 18:2 to 18:3 were more or less evenly distributed among the

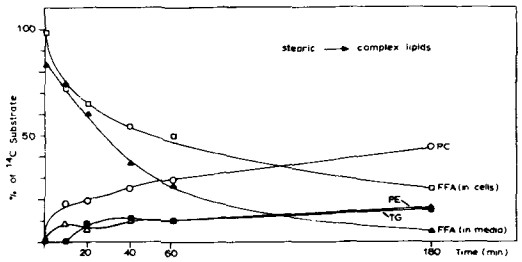


FIG. 1. Uptake and metabolism of ^{14}C-stearic acid by soybean suspension cultures as a function of time. Details of the analysis are described in Experimental Procedures section. TG, triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; FFA (in media), free fatty acid in media; FFA (in cells) self explanatory. Time "O" is the operational term defined in Table III.

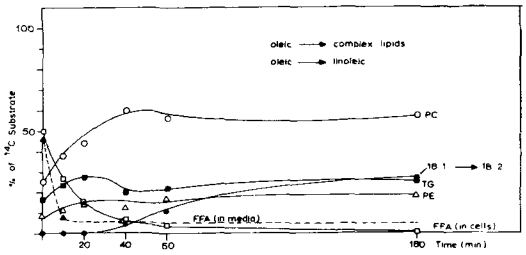


FIG. 2. Uptake and metabolism of ^{14}C-oleic acid by soybean suspension cultures as a function of time. See legend of Figure 1 for definition of symbols.

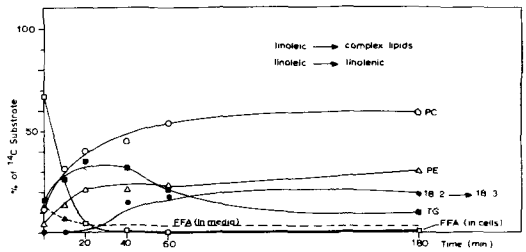


FIG. 3. Uptake and metabolism of ^{14}C-linoleic acid by soybean suspension cultures as a function of time. See legend of Figure 1 for definition of symbols.

principally labeled complex lipids while the FFA remained unlabeled (Table IV). The observation that TG as well as PC and PE were labeled very early in the incubation period can be reasonably explained by assuming that a common pool of oleyl, linoleyl, or linolenyl-CoAs were first formed and were then transferred to complex lipids.

Kinetic measurements of FFA uptake by soybean suspension cultures were complicated by the initial phase of uptake which may involve both a physical, nonenzymic, nonspecific adsorption of FFA from the media and/or a

TABLE IV

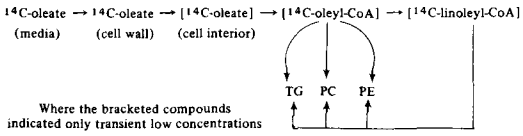
Conversion of ^{14}C-Oleic to ^{14}C-Linoleic Acid by Soybean Suspension Cultures and the Distribution of the Fatty Acids into Complex Lipids as a Function of Time[a]

Complex lipids	Time (min)	% ^{14}C in lipid	% ^{14}C-18:1	% ^{14}C-18:2
Triacylglycerols	30	17	91	9
	60	12	67	33
	120	14	60	40
Free Fatty Acids	30	7	100	tr[b]
	60	6	100	tr[b]
	120	4	94	6
Phosphatidylethanolamines	30	22	86	14
	60	26	74	26
	120	27	63	37
Phosphatidylcholines	30	54	79	21
	60	56	67	33
	120	55	59	41

[a]See Experimental Procedures for conditions of experiment.
[b]tr = trace.

simultaneous movement of FFA into the cell where very rapid subsequent conversion processes occurred presumably in the plasma membrane and/or in the organelle membranes of the cytoplasmic compartment. Data that are not described here show no saturation kinetics in terms of FFA uptake into the cell suggesting no carrier-mediated mechanism at the concentrations employed. Since high concentrations of FFA may markedly modify binding sites or alter membrane characteristics because of their detergent action, these concentrations were avoided and only low concentrations in the range of 1-20 x 10^{-6} M were employed in these studies.

If one assumes that the binding of FFA by suspension cells serves as a marker for its association with nonspecific binding sites on the cell wall; that the activation of FFA is the property of membrane systems in the interior of the cell; that the appearance of the acyl moiety in TG, PC, and PE must reflect the formation of acyl-CoAs and their transfer to the appropriate acceptors; and that the appearance of the desaturation product of the added FFA is simultaneous in the three complex lipids over a given time span, then the following sequence is suggested to explain the results observed in Figures 1-3:



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SHORT COMMUNICATIONS

The Effect of Temperature on the Fatty Acid and Phospholipid Composition of *Cephalosporium falciforme* and *Cephalosporium kiliense*

ABSTRACT

The effect of temperature on the lipid composition of *Cephalosporium falciforme* and *Cephalosporium kiliense*, causative agents of maduromycosis, was investigated. The fungi were grown at 28.5 C and 37 C in a chemically defined medium. The lipids were solvent extracted, purified on Sephadex, and separated into their component classes by silicic acid column chromatography. Five lipid classes were found: (a) sterol esters, (b) triacylglycerides, (c) free fatty acids, (d) sterols, and (e) phospholipids. Fatty acids were analyzed by gas liquid chromatography and phospholipids by thin layer chromatography. Temperature induced changes of varying degrees occurred in both the fatty acid and phospholipid fractions of each organism.

INTRODUCTION

In general, the lipid composition of fungi is affected by the growth temperature employed. Most of the work reported indicates that microbial fatty acid unsaturation increases with a decrease in temperature (1,2).

The selection of fungal pathogens in the present study was prompted by the observation that certain lipid components appear to play a role in the infectious disease mechanisms of some pathogenic microorganisms (3,4). This investigation reports the effect of temperature on the fatty acid and phospholipid composition of two filamentous fungi, each of which has been reported as a causative agent of maduromycosis.

MATERIALS AND METHODS

Cultures of *Cephalosporium falciforme* and *C. kiliense* were grown in a chemically defined medium (5) at 28.5 C and 37 C. The cells were harvested at the time of maximal growth, washed, and lyophilized. The lipids were extracted from the lyophilized cells by the method of Huston and Albro (6), purified on Sephadex G-25 (7), and fractionated by silicic acid chromatography (8). Individual fractions were quantitated gravimetrically and purity was determined by thin layer chromatography (TLC) on Silica Gel H. After saponification and hydrolysis of the total lipid sample (9), the fatty acids were methylated (10) and analyzed by gas liquid chromatography using 16.5% diethylene glycol succinate on Anakrom ABS (100-110 mesh). Phospholipids were identified by means of TLC using the solvent system chloroform:methanol:water 65:25:4 v/v/v (11) and appropriate standards. After development by exposure to iodine vapors, the visible spots were recovered and quantitated according to phosphorus content (12).

RESULTS AND DISCUSSION

Maximal growth was obtained after 24 hr incubation at 28.5 C for both organisms. At

TABLE I

Weight Percentages of Lipid Classes of *Cephalosporium falciforme* and *C. kiliense* Grown at 28.5 C and 37.0 C

	C. falciforme		*C. kiliense*	
Lipid class	28.5 C	37.0 C	28.5 C	37.0 C
Sterol esters	9	13	16	10
Triacylglycerides	25	22	18	22
Free fatty acids	4	3	3	4
Sterols	13	12	14	14
Phospholipids	49	50	49	50
Recovered sample	5.28 mg	5.57 mg	6.06 mg	5.74 mg
Total sample	5.45	5.92	6.48	6.12
Recovery	96.0%	94.1%	93.5%	93.8%

TABLE II

Phospholipid Composition of *Cephalosporium falciforme* and *C. kiliense* Grown at 28.5 C and 37.0 C (μg Pi)[a]

Phospholipids	*C. falciforme*		*C. kiliense*	
	28.5 C	37.0 C	28.5 C	37.0 C
Phosphatidyl ethanolamine	1.36	1.20	1.25	0.88
Diphosphatidyl glycerol	0.54	0.55	0.46	0.31
Phosphatidyl choline	4.50	2.80	4.15	3.35
Phosphatidyl serine	0.82	1.82	1.13	0.70
Sphingomyelin	1.20	0.78	0.30	0.40
Lysophosphatidyl choline	0.65	1.20	0.95	-
Recovered sample	9.07 mg	8.35 mg	8.24 mg	5.64 mg
Total sample	8.95	7.80	8.50	5.95
Recovery	101.3%	107.1%	96.9%	94.8%

[a](μg Pi) = micro g phosphorus.

TABLE III

Relative Percentages of Total Fatty Acid Content of *Cephalosporium falciforme* and *C. kiliense* Grown at 28.5 C and 37.0 C

Fatty acid	*C. falciforme*		*C. kiliense*	
	28.5 C	37.0 C	28.5 C	37.0 C
10:0	1.6	1.8	1.6	3.9
11:0	0.3	0.8	0.5	1.3
11:1	0.6	0.7	0.8	0.9
12:0	0.4	0.8	0.4	1.6
12:1	-	-	-	1.4
13:0	0.5	0.9	0.4	2.0
14:0	1.8	1.6	1.0	3.0
14:1	0.2	1.0	0.2	0.3
15:0	1.2	1.0	tr[a]	0.5
15:1	0.5	0.4	0.6	1.9
16:0	16.9	14.3	15.5	10.0
16:1	1.8	1.4	1.4	3.1
17:0	1.0	1.2	-	-
17:1	1.0	1.0	0.6	2.3
18:0	4.7	3.8	6.4	4.2
18:1	13.5	13.0	42.4	33.2
18:2	25.8	41.7	24.4	22.2
18:3	17.4	11.1	3.2	3.3
20:0	0.3	-	0.4	2.9
21:0	4.5	3.4	0.2	2.0
21:1	6.0	-	-	-
Total saturated	33.2	29.6	26.4	31.4
Total unsaturated	66.8	70.3	73.6	68.6

[a]tr = trace (less than 0.1%).

37 C greatest growth yields occurred after 72 hr with *C. falciforme* and after 48 hr with *C. kiliense*. A total of 26 mg of lipid per gram of lyophilized cells was recovered from both organisms grown at 28.5 C. At 37 C, *C. falciforme* yielded 18 mg and *C. kiliense* 16 mg, respectively. Low lipid yields of less than 5% of the total weight, although uncommon in fungi, have been reported for several species of Phycomycetes and Ascomycetes (1). A likely contributing factor to the levels of lipid obtained in the present study was the utilization of a chemically defined medium rather than a complex, enriched substrate.

In the present investigation, temperature related changes in the lipid classes detected in both fungi were comparatively slight (Table I). Of interest is the fact that the phospholipid

fraction comprised one half the total lipid sample at each temperature employed. Consequently, this lipid class was investigated further. The phosphoglycerides found in both organisms (Table II) have also been detected in other fungi (1). A single sphingolipid corresponding to sphingomyelin, as determined by TLC, was detected in both species of *Cephalosporium*. Sphingomyelin, which is not commonly reported in fungal lipids has, nevertheless, been detected in another pathogenic fungus, *Trichophyton rubrum* (13). The occurrence of sphingolipids in fungi warrants our interest in view of the association of this class of lipids with several human disorders (14). Also noteworthy in the present investigation is the absence of phosphatidyl inositol in either fungus since this phosphoglyceride is known to occur in a wide variety of fungi (1).

The fatty acids of the total lipid samples of both fungi grown at each temperature are shown in Table III. The major acids detected correspond, in general, to those reported for other fungi (1,15). With respect to the fatty acids detected, *C. kiliense* conformed more closely to the often reported phenomenon of increased unsaturation at lower temperatures. *C. falciforme*, on the other hand, displayed a tendency in the opposite direction although the magnitude of the change in unsaturation was small. Of particular significance was the presence of fatty acid 21:0 in both test species. In addition, *C. falciforme* contained a 21:1 component, in a concentration of 6%, after growth at 28.5 C. Although 21:0 has been reported in *Ceratocystis fagacearum* (16) and *Fomes igniarius* (17) and the 21:1 acid in *Amanita muscaria* and *A. rubescens* (18), these fatty acids represent relatively unusual components of fungal lipids.

From the results obtained, it is obvious that both qualitative and quantitative alterations occurred, as a function of temperature, in the lipids of the fungal pathogens investigated. A direct correlation between mouse virulence and the amount of lipid present in *Blastomyces dermatitidis* has already been reported (19). In view of this observation, it would be of considerable interest to determine the part, if any, the temperature related lipid components of *C. falciforme* and *C. kiliense* may assume in the causation of disease.

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1-Docosanol and Other Long Chain Primary Alcohols in Developing Rat Brain

ABSTRACT

Long chain alcohols were detected in developing rat brain at ages ranging from 5 to 40 days. They were at their highest level of 0.0109% of the total lipids at the age of 10 days and decreased to 0.0036% at the age of 40 days. They consisted mainly of hexadecanol, octadecanol, octadecenol, eicosanol, docosanol, and tetracosanol. The fact that substantial amounts of fatty alcohols having more than 20 carbon atoms were present in myelinating rat brain indicated a chain length specificity in their utilization for *O*-alkyl and *O*-alk-1-enyl glycerolipid biosynthesis.

INTRODUCTION

Fatty alcohols are important intermediates in the biosynthesis of *O*-alkyl and *O*-alk-1-enyl glycerolipids (1). Small amounts of alcohols varying in chain length from C_{16} to C_{22} were detected in adult mammalian brain and heart (2,3), and somewhat larger amounts in transplantable neoplasms (3). Their biosynthesis from the corresponding fatty acids was demonstrated in vitro in a variety of mammalian (4-6) and nonmammalian (7-9) systems.

Developing rat brain was shown to produce hexadecanol from palmitic acid, both in vivo (10,11) and in vitro (6), and the rate of this reduction was highest at the age of 15 days (6). In rat brain, phospholipid biosynthesis reaches highest levels during myelination between the ages of 10 and 20 days. Thus, it was of interest to determine the levels of free alcohols present in the brain during this period and to compare their composition with that of the *O*-alkyl and *O*-alk-1-enyl glycerols.

In the present communication, we report the amounts and composition of fatty alcohols in developing rat brain between the ages of 5 and 40 days. Their composition did not correspond to that of the *O*-alkyl and *O*-alk-1-enyl glycerols. The fact that relatively large amounts of alcohols having more than 20 carbon atoms were present during myelination indicates a distinct chain length specificity in their utilization for *O*-alkyl and *O*-alk-1-enyl glycerolipid biosynthesis.

EXPERIMENTAL PROCEDURES

Male albino rats (ARS Sprague Dawley, Madison, WI), 5 to 40 days old, were killed by decapitation in groups of 7 to 20, brains from each group were pooled and the lipids were extracted (12). To each of the lipid extracts, 50 μg of 1-heptadecanol was added as internal standard. Lipids from an additional group of 20-day-old rats were analyzed without adding the internal standard in order to establish the level of heptadecanol among the brain alcohols.

In each case, the lipids were fractionated on layers of Silica Gel H (Merck), 3 mm thick (13), essentially as described previously (2), but using hexane:diethyl ether:acetic acid 40:60:1 v/v/v as developing solvent. The leading edge of the cholesterol fraction, including the fatty alcohols, was scraped off and eluted with chloroform:methanol 4:1 v/v. This crude alcohol fraction was refractionated by thin layer chromatography (TLC) on layers, 0.5 mm thick, and the fatty alcohols were eluted as described above.

Preparation of the alkyl acetates and analysis by gas liquid chromatography (GLC) were as described previously (2) using ethylene glycol succinate (10% EGSS-X on Gas Chrom P, 100-120 mesh) at 190 C and silicone gum rubber (SE-30 on Anachrom ABS, 90-100 mesh) at 220 C. Weight percentages of alcohols were calculated by triangulation of peak areas;

TABLE I

Fatty Alcohol Concentration in Developing Rat Brain

Age (days)	Number of rats	Total brain weight (g)	Total lipids (mg/g wet tissue)	Fatty alcohols (% of total lipids)	Fatty alcohols (nmol/g wet tissue)
5	20	9.61	24.5	0.0071	6.52
10	10	9.30	35.2	0.0109	14.03
15	10	11.72	45.6	0.0071	12.34
20	10	11.48	50.9	0.0065	12.26
40	7	8.47	68.8	0.0036	9.42

TABLE II

Fatty Alcohol Composition in Developing Rat Brain

Age (days)	Composition (wt %) of alcohols[a]					
	16:0	18:0	18:1	20:0	22:0	24:0
5	28.8	46.9	8.9	5.0	10.4	trace
10	24.6	35.3	13.7	3.5	22.9	trace
15	34.7	45.7	10.6	3.1	5.9	trace
20	54.9	25.9	6.4	1.5	6.1	5.2
40	42.0	33.5	11.1	2.7	9.3	1.4

[a]Traces (<0.5%) of 15:0, 17:0, 19:0 and 21:0 were also detected.

absolute amounts were determined by comparison of peak areas with that of the internal standard.

Trimethylsilyl (TMS) ethers of the brain alcohol fraction as well as of synthetic standards were prepared by reaction with hexamethyl disilazane and trimethylchlorosilane in dry pyridine (14). The TMS derivatives were analyzed on an LKB gas chromatograph-mass spectrometer, Type 9000. The GLC column (3% OV-1) was operated from 150 C to 300 C at a temperature program of 8 C/min. Mass spectra were taken at 20 eV and 70 eV.

RESULTS

Long chain alcohols were found in developing rat brain in the amounts listed in Table I. They were at their highest level of 0.0109% w/w of the total lipids at the age of 10 days, the beginning of myelination.

With increasing age the total amount of free alcohols present in rat brain decreased to 0.0036% w/w of the total lipids, which is comparable to the amount of 0.002% found previously for adult bovine and porcine brain (2). On the basis of total brain tissue, this decrease was less pronounced due to the concomitant increase in total brain lipids.

The relative amounts of the individual fatty alcohols of rat brain are listed in Table II. As is evident from Table II, substantial amounts of 1-docosanol were present among the fatty alcohols at all ages examined, reaching a maximum at the age of 10 days.

Preparation of the TMS ethers of the alcohol fraction from the 20-day-old rat brain and analysis by GLC-mass spectrometry confirmed their structures. Comparison of the spectra obtained from TMS ethers of synthetic 1-hexadecanol and 1-docosanol with those obtained from the appropriate GLC fractions of the natural mixture showed identity of the diagnostic peaks. All long chain alcohols showed molecular ions of low intensity but very intense M-15 ions which represented the major peaks in the high mass unit region of each spectrum.

DISCUSSION

The presence of significant amounts of long chain alcohols of chain lengths C_{20} to C_{24} in developing rat brain is of interest because the corresponding *O*-alkyl or *O*-alk-1-enyl moieties are not found among the glycerophosphatides of mammalian brain (15). Analysis of the *O*-alk-1-enyl glycerols derived from the total lipids of each group of animals used in this study confirmed the fact that mainly $C_{16:0}$, $C_{18:0}$, and $C_{18:1}$ structures were present with only traces of $C_{20:0}$ homologues. Since ether lipid biosynthesis is proceeding at a high rate during the period of myelination, we postulate a distinct chain length specificity for the biosynthesis of *O*-alkyl glycerolipids. In contrast, previous findings demonstrated a lack of specificity with regard to certain structural features of the long chain alcohols. The presence of additional double bonds (16) or certain functional groups (17,18) in primary alcohols ranging in chain length from C_{16} to C_{18} did not impede their incorporation into *O*-alkyl glycerolipids.

Although some fatty alcohols could be formed in rat brain by degradation of sphinganine (19) or *O*-alkyl glyerol (20), those actually used for ether lipid biosynthesis during myelination are apparently produced by reduction of fatty acids (21). Our data imply that this reduction involves mainly saturated and certain monounsaturated fatty acids ranging from C_{16} to C_{24}.

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Lipid Metabolism in Early Development Using Labeled Precursors Incorporated during Oogenesis and in Cell-Free Embryo Homogenates

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ABSTRACT

Embryos of the toad, *Bufo arenarum, Hensel*, taken during early stages of development were used to survey the [U-^{14}C] glycerol and ^{32}P lipid labeling. When precursors were supplied at the time of oogenesis, large differences in specific activities of phospholipid were observed. Using ^{32}P, a steep rise as a function of development was evidenced. Triglycerides contained much higher proportions of [U-^{14}C] glycerol than phospholipids when administered to the female toad along with a pituitary homogenate. However, lack of [U-^{14}C] glycerol uptake into lipids was observed when cell-free homogenates of eggs at different stages of development ranging from unfertilized oocyte to mid-gastrula were incubated in unsupplemented amphibian Ringer. At the later stage, significant de novo biosynthesis of lipids from glycerol began to be measurable, whereas during cell cleavage intracellular redistribution of preformed phosphoglycerides was used for membrane assembly.

INTRODUCTION

Lipid metabolism during oogenesis and early development has not been explored in detail. Our previous studies using ^{32}P have disclosed an increased labeling of phospholipids of the entire embryo and of the postmitochondrial supernatant, the mitochondrial fraction, and, to a lesser extent, the yolk platelet fraction (1). A decrease in acid-soluble phosphate radioactivity was observed. It was not clearly assessed if the changes in ^{32}P imply de novo phospholipid biosynthesis or a turnover of the polar moiety. The present paper deals with the [U-^{14}C]- glycerol and ^{32}P uptake into lipids during oogenesis and in early developing embryos.

MATERIALS AND METHODS

Animal Experiments

Adult toads (*Bufo arenarum, Hensel*) were employed. Females weighing ca. 120-150 g were injected simultaneously with a pituitary extract and 40 μCi of [U-^{14}C] glycerol (uniformly labeled, New England Nuclear Corp., Boston, MA., specific activity 7.4 mCi/mM) into the dorsal lymphatic sacs.

Between 16-20 hr after the injection, females began to eliminate oocytes through the cloaca, and at that time animals were killed and the ova collected from the ovisacs. Fertilization was accomplished by adding an homologous testes homogenate, and development was allowed to proceed in a well aerated amphibian Ringer solution. The amount of radioactivity injected did not alter the embryonic development, which proceeded according to the morphological tables of Del Conte and Sirling (2).

Twenty five hundred oocytes or embryos from an unlabeled ovulation were sorted out and homogenized with a Potter-Elvehjem in 30 ml of Tris Buffer 50 mM pH 7.4 containing 1 mM EDTA, 11.1 mM NaCl, 0.13 mM KCl, and 0.27 mM Ca_2Cl. Fifteen μCi of [U-^{14}C] glycerol were added and rapidly shaken, then a 2.5 ml aliquot was taken out and dropped in chloroform-methanol (3) as zero time. The remaining homogenate was incubated at 27 C and 2.5 ml aliquots were taken at the desired time and treated as above.

Lipid Analyses

Oocytes and embryos were dejellied by brief exposure to neutralized 2% thioglycholic acid, counted, and then homogenized in chloroform: methanol 2:1 according to Folch et al. (4).

In in vitro experiments, lipid extraction was carried out following the Bligh and Dyer (3) method. After removing the proteins from the interphase, two extractions with chloroform: methanol 2:1 were performed. Chromatographic separations on 500 μ layers of Silica Gel G were carried out. Samples were applied in 8-10 cm bands and developed with hexane: diethyl ether:acetic acid (60:40:2.5). From the origin to the solvent front, the following bands were scraped off: phospholipids, X fraction, cholesterol, diacylglycerols, free fatty acids, tri-

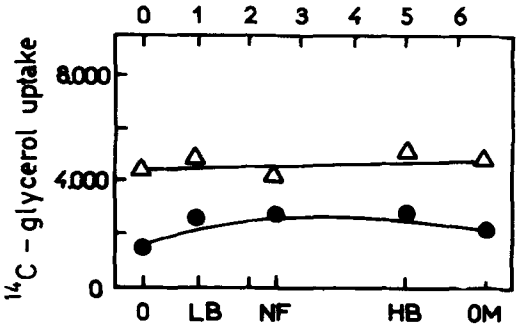

FIG. 1. [U-^{14}C] glycerol incorporation (CPM) in phospholipid and triglycerides during early development. Each point represents samples containing 1000 oocytes or embryos; phospholipid and triglycerides were separated as described in methods. The abbreviations on abscissa represent the following 0: oocyte; LB: Late blastula; NF: neuralfold; HB: heart beat; OM: open mouth. The numbers across the top stand for days of development.

acylglycerols, and cholesterol esteres. Phospholipid phosphorous was determined after perchloric acid digestion according to Rouser et al. (5).

Radioactivity of total lipid extracts was measured on a Packard Tricarb liquid scincillation counter, using 5 ml of 4% Omnifluor (New England Nuclear Corp., Boston, MA) in toluene. Individual lipids were counted after scraping the silica gel into vials containing 10 ml of the same liquid scintillator.

RESULTS

Labeling of Toad Embryos by [U-^{14}C] Glycerol Taken up during Oogenesis

When [U-^{14}C] glycerol was administered to the female toad along with the pituitary homogenate, endogenously labeled oocytes were obtained. Both polar and neutral lipids were labeled with [U-^{14}C] glycerol in the unfertilized oocyte. In Table I, it can be seen that 69% of the radioactivity was found in triglycerides

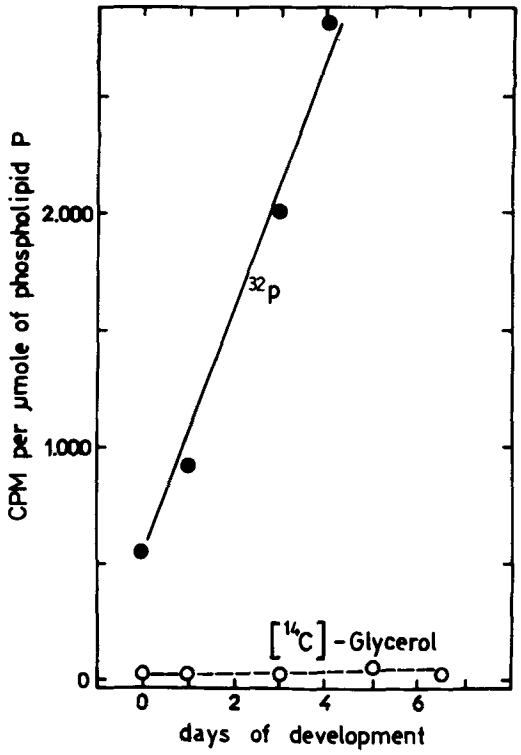

FIG. 2. In vivo uptake of precursors in entire early developing toad embryos. The ^{32}P incorporation was measured in washed total lipid extracts of eggs labeled during induction of ovulation by injecting 150 µCi of sterile radioactive ortho phosphate, pH 7.4 ($Na_2H^{32}PO_4$ 74.28 Ci/g P) per 100 g of body weight. [U-^{14}C]-glycerol incorporation was measured in chromatographically isolated phospholipids.

whereas phospholipids contained 24%. Only a very small amount of radioactivity was recovered in free fatty acids or diglycerides. A band (fraction X) with thin layer chromatographic properties similar to monoglycerides was also labeled. As egg development proceeded, the labeling distribution was not significantly modified (Figure 1 and Table I).

TABLE I

Percentage Distribution of U-^{14}C Glycerol in Lipid Fractions during Early Development In vivo[a]

Lipid fractions	Unfertilized oocyte	Late blastula	Neural fold	Heart beat	Open mouth
Phospholipids	23.7	32.2	32.5	35.1	28.4
X fraction	4.8	5.2	16.0	-	1.6
Cholesterol	-	-	-	-	-
Diglycerides	1.7	1.4	-	-	1.0
Free fatty acids	0.5	0.1	-	-	0.3
Triglycerides	69.2	61.2	51.4	64.9	68.7

[a]Figures represent percent of total counts recovered after thin layer chromatography (TLC) separation.

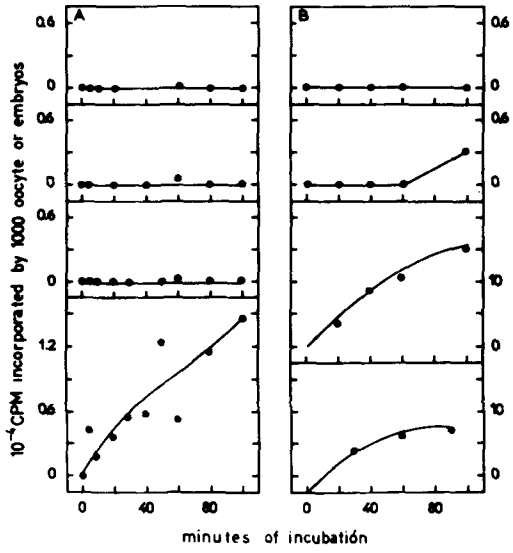

FIG. 3. In vitro labeling by [U-^{14}C] glycerol of cell-free embryo lipids. In experiment A, 15 μCi of [U-^{14}C] glycerol per 2500 oocytes or embryos were added to the incubation media. In experiment B, 15 μCi of [U-^{14}C] glycerol per 1800 oocytes or embryos were added. Each point represents one sample. From top to bottom, the boxes stand for the following stages: surveyed:unfertilized oocyte, late blastula, early-gastrula, and neural tube (experiment A); and unfertilized oocyte, early gastrula, tail bud, and heart beat (experiment B).

Comparative Uptake of ^{32}P and of [U-^{14}C] Glycerol into Phospholipids in early Developing Embryos

In Figure 2 are presented results from two separate experiments depicting the incorporation of [U-^{14}C] glycerol and ^{32}P in phospholipids as a function of embryogenesis. When the radioactivity was expressed per μmole of phospholipid P, a higher value was obtained for ^{32}P than for [U-^{14}C] glycerol in unfertilized oocyte. A slight increase for [U-^{14}C] glycerol was observed during egg development, but specific activity for ^{32}P rose steeply.

In vitro Incorporation of [U-^{14}C] Glycerol into the Lipids of Cell-free Embryo Homogenates

In lipids of cell-free homogenates of unsupplemented, unfertilized oocytes, undetectable [U-^{14}C] glycerol uptake was found (Figure 3). A similar result was obtained after middle gastrula. In Figure 3, data are presented showing that embryos from neural tube stage onwards take up the labeled precursor. Most of the radioactivity was recovered in the phospholipids at 40 and 60 min of incubation. At 20 min, however, triglycerides contained 14% of the total (Figure 4).

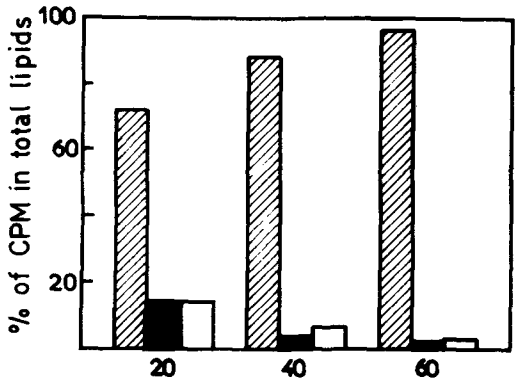

FIG. 4. Percentage uptake of [U-^{14}C] glycerol into lipids by tail bud embryo in vitro. Cross-hatched bar: Phospholipid; Black bar: Triglycerides; Open bar: a fraction that runs as monoglycerides.

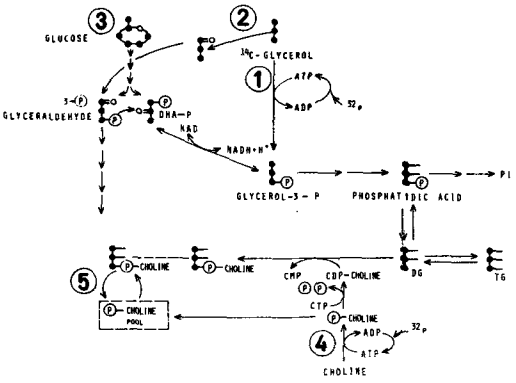

FIG. 5. Diagram showing the possible pathways involved in phospholipid synthesis during early embryonic development.

DISCUSSION

Contrasting differences were found between the lipids of early developing toad embryos when [U-^{14}C] glycerol was incorporated during gonadotropin-induced oogenesis or when added to cell-free homogenates. In the former case, triglycerides and phospholipids were labeled by the precursor, and the incorporation into the neutral glycerides was much higher. This profile was maintained throughout the developmental stages studied implying that the labeled glycerol was taken up during oogenesis in either of two ways, by direct uptake of the glycerol into the maturating oocyte or by uptake and conversion by the liver prior to its deposition in the oocyte. This second possibility seems more likely and involves lipid biosynthesis by the toad liver using [U-^{14}C] glycerol as a precursor and then transfer through the blood stream to the ovary. Grant (6) arrived at a similar conclusion using ^{32}P as precursor and related ^{32}P

incorporation to surface area in the mature oocyte.

Amphibian liver is equipped with the necessary enzymes to perform lipid biosynthesis (7) and, during ovulation, the transfer from the liver into the ovaries of lipophosphoproteins takes place (8). The uptake of entire phospholipids from the blood stream does, in fact, occur in cellular membranes of rat tissues (9). Until late gastrulation, U-^{14}C glycerol uptake into lipids does not take place. However, from neurulation onwards lipid labeling was evidenced. Most of the radioactivity was recovered in the phospholipids, and the triglycerides contained only about 3% of the label after 60 min of incubation of tail bud embryo homogenate.

A different U-^{14}C distribution in lipids was observed in homogenates compared with the embryos prelabeled during oogenesis. This may indicate that only after middle gastrula does the embryo acquire the ability to use U-^{14}C-glycerol as lipid precursor, and that during oogenesis only the uptake of preformed lipids occured. Thus, there may also take place the appearance of an appropriate ionic environment for lipid enzymes, or the de novo biosynthesis of certain key enzymes for lipid formation or both. At the time of gastrulation, several important events occur, such as Ca^{++} release (10) and activation of transcription (11).

Until mid-gastrula at least, polar lipid biosynthesis may be restricted or absent despite the great requirements of lipids for plasma membrane formation during cell cleavage. We have previously suggested that, in early embryogenesis, an intracellular transfer of phospholipids may occur between a storage site, yolk platelets, and the membranes being assembled (12,13). This proposal was the first to imply a physiological role of the phospholipid exchange between subcellular structures (1, 12, 13, 16, 17, 19). The beginning of incorporation of [U-^{14}C] glycerol into phospholipids at gastrula stage may mean that the appropiate enzymes appear and that phospholipids are still supplied to membranes from yolk platelets.

The route of glycerol utilization from neurula onwards remains to be established. It may involve steps 1 or 2 (Figure 5) but the former is the more likely possibility since, in adult liver, the Km of glycerol kinase is several-fold lower than that for glycerol dehydrogenase (14,15). The actual contribution of glycolysis to lipid biosynthesis in embryos (3 in Fiture 5) is being studied at the present time in our laboratory. The recycling of glycerol may not be great since most of the label was found in the appropriate moiety of lipids.

The differential labeling observed with ^{32}P and [U-^{14}C] glycerol indicates that these precursors are unequally metabolized in early embryos. If the increase in ^{14}C labeling from fertilization onwards reflects de novo phospholipid biosynthesis, an exceedingly higher rate of ^{32}P turnover is also apparent (Figure 2). Thus, choline kinase (18) through the cytidine nucleotide pathway and turnover of the polar moiety of phospholipids may be responsible for the described differences (4 and 5 in Fig. 5). Choline kinase has been found to be increasingly active during the early embryogenesis of the sea urchin (18). Preliminary evidence from this laboratory indicates that triacylglycerols may contribute towards the biosynthesis of phospholipids in early developing enbryos supplying diacylglycerols.

ACKNOWLEDGMENT

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Effect of a Diet Rich in Sunflower Oil on Aspects of Lipid Metabolism in the Genetically-Obese Rat

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ABSTRACT

Aspects of the lipid metabolism of male, obese and lean Zucker rats were compared using animals which had been fed ad libitum for 32 days on a diet (HS) which contained 200 g sunflowerseed oil/kg or one (LS) which contained 50 g/kg of the oil. When compared with the LS diet, the HS diet decreased the characteristic lipid accretion in the liver of obese rats from 126 mg (LS) to 81 mg (HS)/g wet weight; corresponding values for the lean rats were 39 mg and 56 mg/g wet weight of liver, respectively. The HS diet depressed lipid synthesis de novo by liver homogenates and decreased the Δ9-desaturase activity of liver microsomes from obese and lean rats by about 50%. Δ9-Desaturase activity in vitro was also depressed by the addition of linoleic acid to liver microsomes from both obese and lean rats fed ad libitum on a standard laboratory diet. Depressed Δ9-desaturase activity, due to ingestion of the HS diet, was reflected in lower ratios of 16:1/16:0 and 18:1/18:0 fatty acids in tissue lipids from obese and lean rats. Ingestion of the HS compared with the LS diet resulted in increased proportions of 18:2ω6 in liver lipids and adipose tissue triacylglycerols of obese and lean rats. The HS diet also increased the proportions of 20:4ω6 in adipose triacylglycerols of obese and lean rats and in liver lipids of obese animals but not in their lean littermates.

TABLE I

Composition (g/kg) of Diets

Ingredient	LS[a]	HS[b]
Sunflowerseed oil	50	200
Sucrose	561	411
Glycerol	150	150
Casein	150	150
Mineral mix[c]	37	37
Trace element mix[d]	2	2
Vitamin mix[e]	50	50

[a]LS = 50 g sunflowerseed oil/kg.

[b]HS = 200 g sunflowerseed oil/kg.

[c]Contained (g): $CaCO_3$ 12.5, Na_2HPO_4 8.6, KH_2PO_4 8.2, KCl 6.0 and $MgCl_2$ 3.4.

[d]Contained (g): KI 0.032, $MnSO_4.4H_2O$ 0.21, $ZnSO_4.7H_2O$ 0.176, $FeSO_4.7H_2O$ 0.26, $CuSO_4.5H_2O$ 0.098 made up to 2 g with sucrose.

[e]Contained (g): thiamine 0.01, riboflavin 0.02, nicotinic acid 0.04, pyridoxine 0.01, pteroylmono-l glutamic acid 0.01, cyanocobalamin 0.005, calcium pantothenate 0.16, myoinositol 0.8, choline chloride 20, p-amino-benzoic acid 0.02, ascorbic acid 0.075, D-α-tocopheryl acetate 0.4, retinyl acetate 0.0034, cholecalciferol 0.004, menaphthene sodium bisulphite 0.001. This was made up to 50 g with sucrose.

INTRODUCTION

Genetically-obese Zucker rats are characterized by an enhanced rate of lipogenesis in the liver and to a lesser extent in adipose tissue (1) resulting in excessive fat accretion in these tissues compared with their lean littermates (2). The tissue lipids of the obese rats also contain greater proportions of palmitoleic (16:1) and oleic (18:1) acids and lower proportions of the polyunsaturated fatty acids linoleic (18:2) and arachidonic (20:4) as compared with lean littermates (3, and K.W.J. Wahle, unpublished observations). These differences in fatty acid composition reflect enhanced endogenous synthesis of saturated fatty acids and their increased Δ9-desaturation in the obese animals (3, and K.W.J. Wahle, unpublished observations). A similar fatty acid composition of tissue lipids is evident in normal rats fed on diets high in carbohydrate and low in fat, particularly when these diets are low in linoleic acid (4-6). Such diets also occasion enhanced rates of lipogenesis in the animals and induce the accumulation of lipid in the liver (5,6). Supplementation of these high carbohydrate diets with fat, particularly fat containing linoleic acid, restores fatty acid synthesis (and presumably Δ9-desaturase activity) and the relative proportions of fatty acids in tissue lipids to values similar to those found in rats fed on stock diets (4-10).

The purpose of the present work, a preliminary account of which has been given (11), was to compare the effect of two diets containing 5% and 20% sunflowerseed oil (65% linoleic acid, i.e., 18:2ω6) on hepatic lipogenesis and Δ9-desaturase activity and on the fatty acid composition of the tissue lipids of obese and lean Zucker rats with a view to modulating, by dietary means, the genetically-determined excessive lipogenesis and fat accretion in the obese animals.

TABLE II

Effect of Dietary Sunflowerseed Oil on Liver Lipid Content and Liver and Carcass Weights of Obese and Lean Zucker Rats

Diet[a]	Total liver lipid content (mg/g wet weight)	
	Obese	Lean
LS	125.6 ± 19.8	37.2 ± 2.2
HS	81.0 ± 8.2	52.0 ± 4.8
	(5)	(5)
	Liver weights (g wet weight)	
LS	20.5 ± 1.1	14.0 ± 1.0
HS	18.7 ± 1.3	12.1 ± 1.4
	(5)	(5)
	Carcass weights (g)	
LS	304.3 ± 2.2	267.7 ± 6.5
HS	356.0 ± 13.5	222.3 ± 4.2
	(5)	(5)

[a]For details of diets see Table I. Means ± S.E.M. are given with number of animals used in parenthesis.

TABLE III

Effect of Dietary Sunflowerseed Oil on [1-^{14}C] Sodium Acetate Incorporation[a] into Total Lipids by Liver Homogenates from Obese and Lean Zucker Rats

Diet[b]	[1-^{14}C] sodium acetate incorporation into lipid	
	Obese	Lean
LS	142.6 ± 18.1	84.1 ± 1.6
HS	65.1 ± 6.6	46.5 ± 5.7

[a]10^{-6} μmoles incorporated/2h/mg protein. For details of procedure, see Wahle (1974).

[b]For details of diets see Table I. Mean values with S.E.M. for three animals are given.

MATERIALS AND METHODS

Two groups of male Zucker rats from the Rowett Institute colony, each of five obese and five lean littermates aged 32 days were used. They were weaned when 24 days old onto a standard (commercial) laboratory diet containing 3% vegetable oil, 20% protein, 50% carbohydrate, minerals and vitamins [Herbert C. Styles (Bewdley) Ltd., Worcestershire, U.K.]. The animals were housed individually in plastic cages which had stainless steel lids and floors. Each group was fed ad libitum for 32 days on either the LS diet (50 g sunflowerseed oil/kg diet) or HS diet (200 g sunflowerseed oil/kg diets) (Table I) and given free access to water.

Animals were killed by CO_2 asphyxiation followed by exsanguination; liver and samples of perinephric and subcutaneous adipose tissue were taken immediately after death.

The preparation of liver homogenates and microsomes, the determination of [1-^{14}C] sodium acetate incorporation into lipids by homogenates, and the assay system for the desaturation of [1-^{14}C] sodium stearate by microsomes have been described previously (12). Extraction of liver lipids and adipose tissue triacylglycerols and the preparation and determination of their component fatty acids (as methyl esters) by gas liquid chromatography were done using the methods of Duncan and Garton (13). Protein concentrations were determined by the method of Miller (14). Sources of chemicals have been given previously (12).

EXPERIMENTAL PROCEDURES AND RESULTS

The content of lipid was greater in the livers of obese rats than of lean rats when they were fed on either the LS or HS diets (Table II). When the rats were fed on the HS as opposed to the LS diet, the lipid content of livers of obese rats decreased by about 35%, whereas that of livers in lean rats increased by about 28%. Liver weights were greater in obese than in lean rats and were not greatly affected by the nature of the diet. Similarly, carcass weights of obese rats

TABLE IV

Desaturase Activity of Hepatic Microsomes from Obese and Lean Zucker Rats Fed LS, HS, and Standard Diets[a]

	Rats	Diets		
		Standard diet	LS	HS
Desaturase activity[b]	obese	37.7 ± 2.8	40.9 ± 4.5	21.5 ± 2.5
	lean	13.4 ± 3.6	19.1 ± 2.0	7.5 ± 1.8
		(3)	(3)	(3)

[a]For details of diets see Table I and Materials and Methods section. Mean values with S.E.M. given with number of animals in parenthesis.

[b]nmol 18:0 fatty acid converted to 18:1/30 min/mg protein = desaturase activity.

TABLE V

Effect of Linoleic Acid in vitro on Hepatic Microsomal Desaturase Activity of Obese and Lean Zucker Rats

		Linoleic acid (nmoles/mg microsomal protein)				
	Rats	0	150	300	600	1200
% of control desaturase activity[a]	Obese	100	105	81.2	10.2 ± 2.8	4.4 ± 0.4
	Lean	100	120	54.0	19.4 ± 5.8	3.1 ± 0.4
		(4)[b]	(2)	(2)	(4)	(3)

[a]% conversion of stearic to oleic/30 min/mg protein.

[b]Means and means ± S.E.M. given for number of observations in parentheses.

were greater than those of lean animals which had been fed on the same diet. However, ingestion of the HS diet as opposed to the LS diet increased carcass weights in obese rats, but decreased carcass weights in their lean littermates by 17%.

Incorporation of [1-^{14}C] sodium acetate into total lipids was greater in liver homogenates from obese rats compared with lean rats, regardless of the type of diet consumed (Table III). However, the extent of incorporation was reduced by about 50% for both obese and lean rats which had been fed on the HS diet as opposed to the LS diet.

Reduced Δ9-desaturase activity was also found in liver microsomes of obese and lean rats fed on the HS compared with the LS diet or standard laboratory diet (Table IV). The standard diet was included in the experiment as a control for the semisynthetic LS and HS diets. Observations on hepatic Δ9-desaturase activity and the fatty acid composition of tissue lipids from obese and lean Zucker rats fed on the standard diet have been reported previously (12). Present observations showed that obese rats had a consistently greater desaturase activity than their lean littermates fed on the same diet. Addition of linoleic acid (300, 600, and 1200 nmol) to incubations containing liver microsomes from either obese or lean rats decreased the Δ9-desaturase activity (Table V).

Ratios of 16:1/16:0 and 18:1/18:0 fatty acids in liver lipids and 16:1/16:0 in subcutaneous triacylglyerols were lower in obese and lean rats given the HS diet as opposed to the LS diet (Table VI). Proportions of linoleic (18:2) and arachidonic (20:4) acids were lower in liver lipids of obese rats than in the liver lipids of their lean littermates when the animals were fed on either the LS or HS diet. Both obese and lean rats, when fed on the HS diet as opposed to the LS diet, had increased proportions of 18:2 in liver lipids, but only in the obese animals was the proportion of 20:4 greater, although values were still lower than in lean rats. When compared with the LS diet, the HS diet also resulted in enhanced proportions of 18:2 and 20:4 in subcutaneous triacylglycerols in both obese and lean rats. However, proportions of these fatty acids were again lower in these lipids from obese rats than in those from their lean littermates given the same diet; proportions of 20:4 were lower in the adipose triacylglycerols than in liver lipids. The fatty acid composition of triacylglycerols from perinephric adipose tissue was similar to that in the subcutaneous tissue and is, therefore, not tabulated.

DISCUSSION

The reduction in the lipid content of livers from obese Zucker rats fed on the HS diet is probably attributable to a depression of the excessive endogenous fatty acid synthesis which occurs in obese compared with lean rats. Enhanced fatty acid synthesis de novo is regarded as a primary cause of the marked hepatic fat accretion in obese rats (1-3). The reduced incorporation of [1-^{14}C] sodium acetate into lipids by liver preparations from obese rats fed on the HS compared with the LS diet supports the above explanation for the reduction in the lipid content of liver in these animals. The excessive hepatic lipid accretion observed in protein-deficient, but otherwise normal, rats which had been fed a protein repletion diet and in normal rats fed a high carbohydrate fat-free diet was also found to decrease when polyunsaturated fatty acids, notably linoleic acid, were included in the diet (5,6,15).

The increase in lipid content of livers from lean rats fed on the HS compared with the LS diet is contrary to the effect observed in obese rats. The reduced incorporation of [1-^{14}C] acetate into lipids by liver preparations indicates a reduced endogenous lipogenesis similar to that observed in obese animals. It is possible, however, that the influx of exogenous fatty

TABLE VI

Effect of Dietary Sunflowerseed Oil on the Fatty Acid Composition (wt %) of Liver Lipids and Subcutaneous Triacylglycerols of Obese and Lean Zucker Rats

Rat	Diet[a]	Gas liquid chromatographic analysis					Ratios	
		16:0	16:1	18:0	18:1	18:2	20:4	16:1/16:0	18:1/18:0
Liver lipids									
Obese	LS	35.3 ± 0.9	9.1 ± 0.5	8.6 ± 0.8	35.9 ± 1.6	4.9 ± 0.5	3.7 ± 0.6	0.26	4.2
	HS	24.3 ± 1.4	4.1 ± 0.6	12.6 ± 0.8	22.5 ± 1.5	19.4 ± 1.9	9.4 ± 1.2	0.17	1.8
Lean	LS	20.8 ± 0.9	3.5 ± 0.4	16.8 ± 0.3	17.2 ± 0.5	14.2 ± 1.1	17.4 ± 1.2	0.17	1.0
	HS	15.6 ± 0.9	0.8 ± 0.1	16.8 ± 1.0	11.4 ± 0.5	26.5 ± 1.7	17.2 ± 1.2	0.05	0.7
Subcutaneous triacylglycerols									
Obese	LS	30.0 ± 0.9	9.5 ± 0.5	4.8 ± 0.2	37.4 ± 0.6	13.4 ± 0.4	0.3 ± 0.03	0.32	7.8
	HS	22.9 ± 0.9	5.2 ± 0.5	3.5 ± 0.2	29.8 ± 0.5	33.5 ± 1.7	0.6 ± 0.0	0.23	8.5
Lean	LS	26.0 ± 0.9	8.1 ± 0.3	3.8 ± 0.1	32.0 ± 0.6	24.4 ± 0.9	0.4 ± 0.02	0.31	8.4
	HS	14.8 ± 0.8	2.0 ± 0.3	3.1 ± 0.1	25.3 ± 0.5	49.6 ± 1.5	0.7 ± 0.04	0.14	8.2

[a]For details of diet see Table I.

acids into the liver of the lean rats masked any effect due to suppression of hepatic fatty acid synthesis and exceeded the capacity of the liver to produce low density lipoproteins. Although the depressed lipogenesis was accompanied by decreased carcass weights in the lean rats fed on the HS compared with those given the LS diet, obese rats had increased carcass weights. Explanations for these differences between the phenotypes are at present obscure.

The Δ9-desaturase activity of a tissue correlates very closely with the fatty acid synthetic activity of the tissue and may be regulated by a common control mechanism (3,16). In this context, the greatly enhanced Δ9-desaturase activity found in hepatic microsomes from obese rats (when compared with lean rats) was depressed to a similar extent as that of lipogenesis de novo by the increase in dietary sunflowerseed oil and the depression was observed in both phenotypes. The major fatty acid in sunflowerseed oil is linoleic acid and the addition of this acid to hepatic microsomes in vitro also resulted in decreased Δ9-desaturase activity.

It has been suggested that polyunsaturated fatty acids, particularly those possessing the ω6 configuration have a specific regulatory effect, both in vivo and in vitro, on fatty acid synthesis and on Δ9-desaturase activity in nonobese strains of mice and rats (4-10,17-21). The effect on lipogenesis in vivo was also found to be independent of the level of dietary carbohydrate (18), an observation that is at variance with a previous suggestion that the effects were due mainly to the decreased proportions of carbohydrate in diets which had been supplemented with fat (22). In this context, dietary saturated fat has actually been reported to increase Δ9-desaturase activity in nonobese rats (19). It seems likely, therefore, that the inhibition of lipogenesis and desaturation in the present investigations attributed to the HS diet could be due to its high content of linoleic acid.

The fatty acid composition of tissue lipids from both obese and lean rats reflected the decrease in Δ9-desaturase activity elicited by the HS diet and also the increased intake of linoleic acid by the animals. The reduced proportions of tissue linoleic and arachidonic acids which is a characteristic of obesity were elevated to values which were similar to or exceeded those found in lean rats fed on the LS diet. The finding that the proportions of 20:4ω6 increased with increased 18:2ω6 in liver lipids of obese rats, but not in those of lean animals, is indicative of differences in the metabolism of 18:2ω6 between the two phenotypes. The proportions of 18:2 and 20:4ω6

fatty acids in the liver of obese rats may be involved, in part, in the aetiology of the abnormal lipid metabolism in these animals if, as has been postulated, these acids have a specific regulatory function in lipogenic tissues (4-10,17-21). In conclusion, it is apparent that the hepatic lipogenesis and desaturase activity in obese rats can be modified by increased dietary sunflowerseed oil in the same way as in lean rats, despite the genetically-determined abnormal lipid metabolism in the obese animals.

ACKNOWLEDGMENT

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Activity of Phospholipid-Synthesizing Enzymes in Rat Liver Plasma Membranes and the Source of Biliary Lecithin[1]

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ABSTRACT

The potential for the synthesis of phosphatidylcholine by the bile canalicular membrane of the liver cell was assessed by measuring the activity of a number of phospholipid synthesizing enzymes in isolated bile canalicular membrane fractions from rat liver. The activity of these various enzymes was compared to that present in noncanalicular liver cell plasma membranes and in microsomes. The CDP-choline:1,2-diacyl-*sn*-glycerol-cholinephosphotransferase was virtually absent from the bile canalicular membranes but the specific activities of S-adenosyl-L-methionine:phosphatidylethanolamine N-methyltransferase and acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase were 11-15% of those found in the microsomes. The bile canalicular membranes also contained detectable acyl-CoA:*sn*-glycero-3-phosphate acyltransferase activity and the ability to potentiate the Ca^{++}-stimulated exchange of bases between different phospholipids. These findings indicate that the bile canalicular membranes have a very limited capacity for the formation of phosphatidylcholine under the assay conditions employed.

INTRODUCTION

It is now well established that the secretion of biliary lecithin is a relatively rapid process which may be dependent upon the simultaneous secretion of bile acids (1-5). Furthermore, the works of Balint et al. (6,7) and of Christie (8) have shown that the fatty acid composition of biliary lecithin is markedly different from that of hepatic lecithin. Biliary lecithin is greatly enriched in palmitic and linoleic acids relative to lecithin derived from liver (6-8). Isotopic studies have failed to show any precursor-product relationship between the total or linoleoyl species of biliary lecithins when compared to the corresponding lecithins from whole liver or isolated microsomal membranes (7,9-11). Although a relationship has been reported between the 1-palmitoyl 2-linoleoyl lecithin in liver and bile when this molecular species has been separated from the 1-stearoyl homologue (12,13), the subcellular origin of biliary lecithin remains an enigma.

The suggestions have been made that biliary phosphatidylcholine may originate from a special hepatic pool of phospholipid (6,7,11) or may be derived from the bile canalicular membrane (BCM) itself (14). The isotopic studies of Yousef et al. (15) have suggested that phospholipid destined for bile could be derived either from microsomal synthesis and subsequent transfer to the BCM or by synthesis in the BCM itself. Subfractionation of the liver cell plasma membranes into a fraction rich in bile canaliculi and the remainder of the liver cell plasma membranes has made it possible to test some of these hypotheses (16,17).

Gregory et al. (11) have reported that CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase is virtually absent from the BCM and suggested a role for this membrane in lipid transport rather than in lipid synthesis. However, lecithin can also be formed by the stepwise methylation of phosphatidylethanolamine using S-adenosyl methionine as the methyl donor (18,19), by the Ca^{++}-stimulated exchange of free choline with the bases of other phospholipids (20), and by the enzymic acylation of lysolecithin (21). This last reaction also appears to be of considerable importance for the rearrangement of the fatty acid composition of that lecithin produced by de novo synthesis (22).

The purpose of the present investigation was to consider the potential of the BCM for phospholipid synthesis. The studies involved the measurement in the isolated BCM of the activity of a variety of enzymes which play a role in phospholipid synthesis.

MATERIALS AND METHODS

Isolation of Membrane Preparations

Male Wistar rats (High Oak Ranch, Toronto, Ontario), weighing ca. 150 g, were used in these studies. Prior to use, the animals were kept in a

[1]A preliminary report of this paper was given at the AOCS Spring Meeting, Dallas, April 1975.

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constant temperature environment (22 C), were in darkness 12 hr each day (7 p.m. to 7 a.m.) and were allowed water ad libitum. For at least 1 wk prior to use, they were maintained on a powdered semisynthetic diet [Teklad Mills, Cat. no: 170125 (modified), Basal Diet with 27% casein and salt mixture USP XIV], and 2 hr immediately prior to sacrifice they were force-fed an amount of diet equal to 1 g/150 g body wt. Liver cell plasma membranes were isolated according to a modification of the method of Song et al. (16) as described by Fisher et al. (17), to obtain one fraction rich in the BCM and the other comprising the remaining plasma membranes (PM). Microsomes were obtained by centrifugation of the postmitochondrial supernatant at 100,000 x g for 1 hr. Samples of the BCM, PM, and microsomal fractions were fixed for 1 hr at 0 C in 2.5% aldehyde buffered for examination by electron microscopy as previously described (16,17).

Enzyme Assay Procedures

Glucose-6-phosphatase (EC.3.1.3.9) activity was measured by the method of Schwartz and Bodansky (23), 5'-nucleotidase (EC.3.1.3.5) by the method of Widnell and Unkeless (24), and leucyl-β-naphthylamidase (EC.3.4.1.1) by the method of Goldbarg and Rutenberg (25). $(Na^+ + K^+)$-activated ATPase (EC.3.6.1.4) and (Mg^{++})-activated ATPase (EC.3.6.1.3) were measured by the method of Boyer and Reno (26).

Assays for phospholipid-synthesizing enzymes were conducted as follows. *CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase* (EC.2.7.8.2) was measured under conditions modified from those previously described (28). The incubation mixture contained 50 mM Tris-HCl buffer (pH 7.4); 610 μM 1,2-diolein; 4.0 mM $MgCl_2$; 24.4 μM CDP-choline-(^{14}C); 0.38 mg Tween-20; and 100 μg of membrane protein in a final volume of 0.5 ml. Incubations were conducted at 37 C for 15 min. All reaction rates were calculated under conditions such that the amount of product formed was linear with respect to time and protein concentration.

The *Ca^{++}-stimulated exchange reaction* for choline incorporation into lecithin was assayed under conditions similar to those employed by Bjerve (20). The incubation medium contained 60 mM imidazole buffer (pH 8.9), 160 μM $CaCl_2$, 150 μM (^{14}C)-choline, and 100-250 μg protein in a total volume of 0.5 ml. The reaction was stopped after 30 min.

The enzyme activity of *acyl-CoA:1-acyl-sn-glycero-3-phosphorylcholine acyltransferase* (EC.2.3.1.23) was measured at 37 C in a medium containing 60 mM Tris-HCl buffer (pH 7.4), 35 μM (^{14}C)-palmitoyl-CoA, 170 μM 1-acyl-*sn*-glycero-3-phosphorylcholine, and 100 μg membrane protein in a total volume of 0.5 ml. The reaction was stopped after 2 min. The lipids were extracted (29) and the rate of (^{14}C)-palmitate incorporation into lecithin was calculated following thin layer chromatography as described by Wittels (30).

The activity of *acyl-CoA:sn-glycero-3-phosphate acyltransferase* was conducted by incubating a mixture of 2.4 mM (^{14}C)-glycerol-3-phosphate, 5.0 μM palmitoyl-CoA, and 200 μg of membrane protein in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4) at 37 C for 2 min. The formation of radioactive lipid was determined as described by Daae (31).

The conversion of *(^{14}C)-glycerol-3-phosphate into lipid* was also assayed in the absence of added acyl-CoA using a generating system. For this latter study, the incubation medium contained 2.4 mM (^{14}C)-glycerol-3-phosphate, 1.0 μM $MgCl_2$, 1.2 mM ATP, 30 μM CoA, and 200 μg of membrane protein in 0.5 ml of 50 mM Tris-HCl buffer (pH 7.4). Incubations were conducted at 37 C for 5 min.

The *S-adenosyl-L-methionine:phosphatidyl-*

TABLE I

Activities of Marker Enzymes in Membrane Fractions from Rat Liver[a]

Enzyme	Microsomes	Bile canalicular membrane	Plasma membrane
Glucose-6-phosphatase	43.0 ± 5.1	0.4 ± 0.1	0.6 ± 0.1
5'-nucleotidase	2.3 ± 0.5	79.8 ± 7.9	21.4 ± 2.41
Leucyl-β-naphthylamidase	0.3 ± 0.1	8.4 ± 1.1	0.5 ± 0.1
(Mg^{++})-ATPase	3.4 ± 0.4	60.0 ± 2.7	34.7 ± 4.8
$(Na^+ + K^+)$-ATPase	0.4 ± 0.1	16.5 ± 1.7	12.5 ± 1.4

[a]All values are given as means ± S.E. from experiments on 12 different membrane preparations. Each preparation was derived from three rats. All enzyme activities are given as μmoles P_i released/mg protein/hr except in the case of leucyl-β-naphthylamidase where activity is expressed as μg β-naphthylamine released/mg protein/hr.

TABLE II

Activity of Enzymes Responsible for Phospholipid Synthesis in Membrane Fractions from Rat Liver[a]

Enzyme reaction	Activity (nmoles/mg protein/hr)		
	Microsomes	Bile canalicular membrane	Plasma membrane
CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase	54.1	0.8	1.2
Ca^{++}-stimulated choline exchange	4.2	1.2	1.6
Acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase	337.2	45.2	41.5
Acyl-CoA:*sn*-glycero-3-phosphate acyltransferase	61.8	5.9	24.5
sn-glycero-3-phosphate incorporation (generating system)	55.5	0.8	17.6
S-Adenosyl-L-methionine:phosphatidylethanolamine N-methyltransferase (minus dimethyl PE)	5.2	0.8	<0.5
S-Adenosyl-L-methionine:phosphatidylethanolamine N-methyltransferase (plus dimethyl PE)	12.7	1.4	<0.5

[a]All values are means from experiments on two to four different membrane preparations. Each preparation was derived from three rats.

ethanolamine N-methyl-transferase (EC.2.1.1.17) was assayed in membrane preparations using conditions modified from those reported by Gibson et al. (32) and LeKim et al. (33). Enzyme activity was measured under optimal conditions both in the absence and presence of added dimethyl phosphatidylethanolamine. In the absence of the exogenous lipid, incubation mixtures contained 120 μM S-adenosyl-L-methionine-(^{14}C) and 0.7 mg of membrane protein in 1.0 ml of 50 mM Tris-HCl buffer (pH 8.2). Incubations were routinely conducted at 37 C for 45 min after which the reaction was stopped and the formation of radioactive lipid was measured (34). In the presence of exogenous lipid, essentially identical conditions were employed with the exception that the incubation medium also contained 0.13 μmoles of dimethyl phosphatidylethanolamine and 0.15 mg of Tween-20 per ml.

The activity of all the different lipid-synthesizing enzymes studied was expressed as nmoles of lipid formed/mg membrane protein/hour. Protein was measured by the method of Lowry et al. (27). All isotopic materials were purchased from the New England Nuclear Corp., Boston, MA.

RESULTS

Examination by transmission electron microscopy of membrane preparations similar to those used in these studies has revealed that the BCM fractions are morphologically similar to those in liver in situ, but scanning electron microscopy has revealed that the BCM consist of a more or less intact tubular network (41). The plasma membranes and the microsomal fractions consist primarily of vesicles and sheets of membranes, essentially free from BCM and other subcellular fractions.

Table I gives the specific activities of the marker enzymes associated with the membranes used in this study. Glucose-6-phosphatase was almost absent from both the BCM and the PM fractions and 5′-nucleotidase; Mg^{++}-ATPase and leucyl-β-naphthylamidase were more concentrated in the BCM than in the PM. The BCM and PM fractions were contaminated to no more than 3% with microsomes based on the relative activities of marker enzymes in the various fractions. More detailed discussion on these marker enzymes has been presented elsewhere (17).

The data in Table II give the activities of various enzymes concerned with phospholipid synthesis as determined under our experimental conditions in the microsomes, BCM and PM. It is clear that *CDP-choline:1,2-diacyl-sn-glycerol cholinephosphotransferase* is virtually absent from the BCM. This observation is in agreement with the findings of Gregory et al. (11) and indicates that the BCM is probably incapable of synthesizing lecithin de novo by the CDP-choline pathway.

The activity of *acyl-CoA:1-acyl-sn-glycero-3-phosphorylcholine acyltransferase* in the BCM and PM was found to be 10-15% of that found

in the microsomes. Previous work by Stein et al. (35) had indicated that the liver cell plasma membranes could acylate lysolecithin to form lecithin at about the same rate as the microsomal fraction. On the other hand, Eibl et al. (36) could find no indication that the plasma membrane of the liver cell had appreciable lysolecithin acyltransferase activity.

The specific activity of *acyl-CoA:sn-glycero-3-phosphate acyltransferase* in the BCM was ca. 10% that of the microsomal fraction while that in the PM was ca. 40%. This indicates that the liver cell plasma membranes and especially their noncanalicular components can acylate glycerol-3-phosphate.

The ability of the BCM and PM to potentiate the *Ca^{++}-stimulated exchange reaction* was found to be 30-40% of that found in the microsomal fraction. It has been reported that this activity predominates in the microsomal fraction (37).

The BCM contained S-adenosyl-L-methionine:phosphatidylethanolamine N-methyltransferase activity which was 11-13% of that found in the microsomes. The PM did not appear to contain any of this enzyme activity, using either endogenous or exogenously added substrate. The absence of N-methyltransferase activity in the PM has been previously reported (38).

DISCUSSION

Although these studies have confirmed the absence of CDP-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase activity in the BCM (11), they have provided evidence which suggests that the BCM can probably synthesize some phosphatidylcholine to a very limited extent by other pathways.

The BCM has activities of acyl-CoA:1-acyl-*sn*-glycero-3-phosphorylcholine acyltransferase, acyl-*sn*-glycero-3-phosphate acyltransferase, and S-adenosyl-L-methionine phosphatidylethanolamine N-methyltransferase which are 10-15% of those found in the microsomes of the same liver. The BCM also has the enzyme activity involved in the Ca^{++}-stimulated exchange of phospholipid bases whereby phosphatidylcholine can be generated.

The fact that only the activity of the S-adenosyl-L-methionine phosphatidylethanolamine N-methyltransferase activity was greater in the BCM than in the PM might suggest that the BCM has no specialized role in phospholipid synthesis and is not, in fact, the origin of biliary lecithin. The enzyme studies were performed under conditions which were optimal for the assay of these enzymes in the microsomes, and these conditions may not be optimal for the BCM and PM. This is particularly possible in the case of the BCM whose membranes are not in the form of sheets and vesicles but in the form of a more-or-less intact tubular network (39). Furthermore, we have preliminary information to the effect that the presence of bile acids increases the activity of certain of these enzymes in the BCM but not in the PM.

centrations, which are involved in the biotransformation of phospholipids and in the synthesis of phosphatidylcholine via pathways other than the CDP-choline pathway. The physiological significance of these enzyme activities remains to be established. If they should prove to be insignificant, it can be concluded that the endoplasmic reticulum is the site of synthesis of the biliary lecithin and that exchange proteins of the type reported by Wirtz and Zilversmit (40) as well as others (41) may provide for the intracellular transport of this lecithin to the BCM.

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Plasma Cholesterol Levels in Suckling and Weaned Kittens, Puppies, and Guinea Pigs

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ABSTRACT

Plasma cholesterol levels in kittens and puppies were low at birth, rose during the suckling period, and then decreased at about the time of weaning. The increase during the suckling period was much greater in puppies than in kittens. No significant differences in plasma cholesterol levels were observed in puppies fed two different types of diet, horse meat or dog chow, after weaning. Guinea pigs had lower plasma cholesterols than either kittens or puppies. The level was highest on the first day after birth, decreased during the next 3 wk, and then remained fairly constant after the animals were weaned.

INTRODUCTION

An earlier report from this laboratory dealt with changes in plasma cholesterol levels observed in several different species of animals during the early weeks of life (1). In general, the values were low at birth, increased rapidly during the first few days of life, remained at elevated levels during the suckling period, and then decreased as the animals were weaned.

Experiments with rabbits (2), rats (3), and calves (1) have indicated that the hypercholesterolemia during the suckling period is primarily due to the ingestion of milk lipids. On the other hand, dietary protein was found to be responsible for the hypercholesterolemia observed in older rabbits fed low fat, cholesterol-free semisynthetic diets (4,5). Elevation of plasma cholesterol was obtained with diets containing milk proteins or proteins from other animal sources, but not with diets containing plant proteins. Addition of 15% by weight of butter to these two types of diet made little difference to the results obtained (4,5).

In view of these findings, it seemed possible that the drop in plasma cholesterol at weaning might be related to the introduction of vegetable proteins into the diet. Since the rabbit and other animal species studied previously (1-3) were either herbivores or omnivores, it seemed of interest to follow the changes in plasma cholesterol levels of young animals from species normally weaned to diets containing a higher proportion of animal protein. Cats and dogs were chosen for this purpose. Guinea pigs were also studied as an example of an herbivore in which the young are born in a highly developed state.

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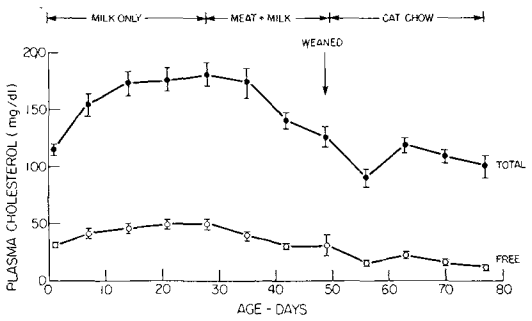

FIG. 1. Plasma cholesterol levels in kittens. Results are given as Mean ± S.E.M. for eight kittens from two different litters. There was no apparent sex difference, so data for the two male and six female kittens were pooled. During the 3 wk prior to removal of the mother, a meat supplement was made available to the kittens. A beef preparation (Romar Pet Supplies, Ltd., Toronto, Ontario) was mixed with a small amount of condensed milk (Carnation Company Limited, Toronto, Ontario) and was placed in a dish in the cage with the mother and litter. According to the manufacturer, the meat preparation contained beef, beef trim, corn meal, and water sufficient to process. Guaranteed Analysis: Protein 12% min., fat 4% min., dry matter 25% max.

METHODS

The cats and dogs used for these experiments were of mixed breeds obtained from a local animal pound. Pregnant females were purchased and maintained in the University animal quarters during the latter part of the pregnancy. The cats were maintained on Purina Cat Chow, and the dogs on Purina Dog Chow. The cat chow contained not less than 30% protein and 8% fat and not more than 12% moisture and 4.5% fibre. The dog chow contained not less than 21% protein and 7% fat and not more than 12% moisture and 4.5% fibre. The guinea pigs were of the English short-hair variety and were obtained from Canadian

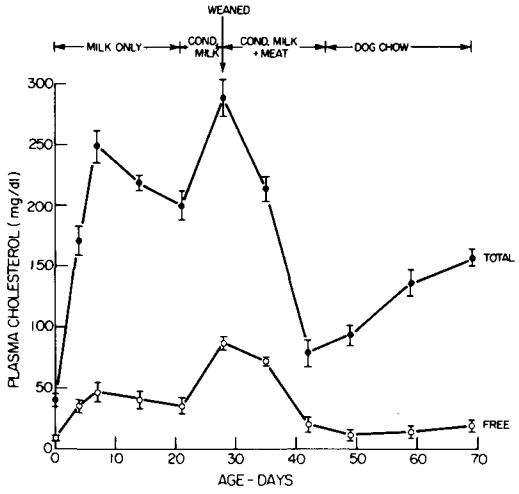

FIG. 2. Plasma cholesterol levels in puppies. Results are given as Mean ± S.E.M. for eight puppies from a single litter (seven male and one female). For a week before the mother was separated from the puppies, they were allowed access to a supplement of condensed milk (Carnation milk diluted with water in a ratio of 3:1). After the mother was removed, a supplement of meat and milk was given (see caption to Fig. 1).

Breeding Laboratories, Montreal, as pregnant females. They were maintained on rabbit pellets (Master Feeds Division of Maple Leaf Mills, Ltd., Toronto, Ont.) with a supplement of carrots and/or lettuce 5 days per week. Details of diets fed to the young on weaning are given in connection with the individual experiments.

Blood for analysis of cholesterol content was obtained from the young animals as follows. The kittens were anesthetized with sodium pentathol or diethyl ether and bled by heart puncture. Ether was preferable for very young kittens up to 4 days of age. No anesthetic was used for the puppies. An area of the neck was shaved and the blood was taken from the jugular vein. The guinea pigs were lightly anesthetized with diethyl ether and bled by heart puncture. After the kittens and guinea pigs were bled, a volume of physiological saline equal to the volume of blood removed was injected intraperitoneally. Heparin was used in all cases to prevent clotting. Free and total cholesterol content of plasma was determined by the method of Sperry and Webb (6).

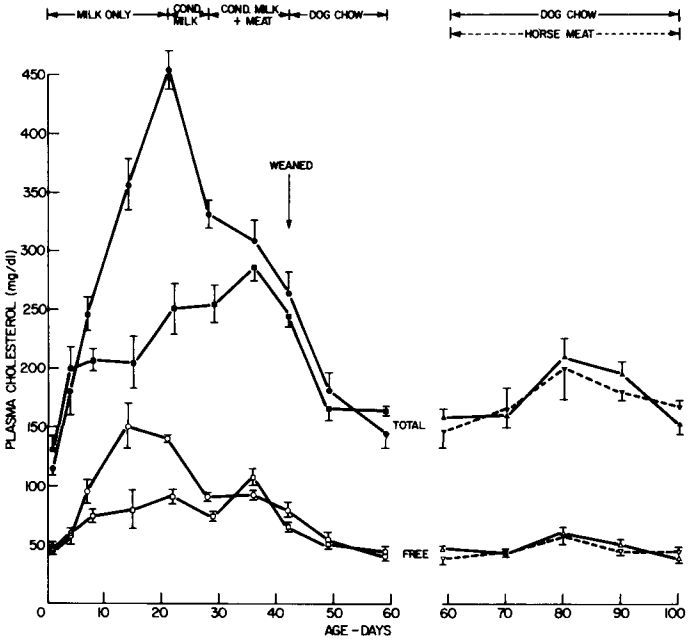

FIG. 3. Plasma cholesterol levels in two litters of puppies. Litter 1 (●—●) consisted of five males and four females, and litter 2 (■—■) of five males and three females. Since there was no sex difference, the data for males and females were pooled. Results are given as Mean ± S.E.M. Both litters were treated alike until they were 59 days old. A total of three males and three females from the two litters were then given a diet of horse meat while seven males and four females continued to receive the dog chow. One male and one female from litter 2 died between 70 and 80 days of age and are not included in this part of the experiment. They were part of the group on dog chow and the deaths were not attributed to the experimental protocol.

RESULTS

Kittens

Variations in the plasma cholesterol levels of kittens from birth until after weaning are shown in Figure 1. The level increased from 115 to 175 mg/dl during the first 2 wk of life and remained at about that level for the next 2 wk. At that point, the kittens were given a daily supplement of meat while continuing to suckle the mother. Over the next 3 wk, the level decreased to 125 mg/dl. The kittens were then weaned to a diet of cat chow, and the plasma cholesterol decreased still more to a level of about 100 mg/dl. Plasma free cholesterol tended to follow the same general trend as plasma total cholesterol (Fig. 1).

The kittens weighed 113 ± 2 g (Mean ± S.E.M.) at birth and gained 795 ± 25 g during the 11 wk of the experiment. Weight gain was continuous and the rate of gain was not affected by weaning.

Puppies

In puppies, there was a more dramatic increase in plasma cholesterol, from 40 to 250 mg/dl, during the first week of life (Fig. 2). The level then fluctuated between 200 and 300 mg/dl until 4 wk of age. At that time, the puppies were weaned to a diet of condensed milk and meat, and over the next 2 wk, the plasma cholesterol fell to less than 100 mg/dl. During the following 4 wk, the puppies were maintained on dog chow, and the plasma cholesterol increased gradually to 150 mg/dl. As with kittens, the plasma free cholesterol tended to rise and fall with the total cholesterol.

In a second experiment involving two litters of puppies, a somewhat different dietary pattern was followed and the study was continued for about 14 wk (Fig. 3). In this experiment, the first blood sample was taken when the puppies were 1 day old and the plasma cholesterols were already in the range of 115-130 mg/dl. They increased rapidly and in one litter reached an average level of 450 mg/dl by the time they were 3 wk of age. Supplements of condensed milk and meat were given over the next 3 wk and at the time of weaning, the average level for both litters was close to 250 mg/dl.

In this experiment, the puppies of both litters were weaned to dog chow and the plasma cholesterols continued to drop to 145-160 mg/dl. When they were 59 days old, six puppies (three males, three females), from the two litters were transferred to a diet of horse meat, and the remaining puppies continued on the chow diet. The results (Fig. 3) showed no significant difference between the plasma cholesterol levels of these two groups over the next 6 wk, at which time the experiment was terminated.

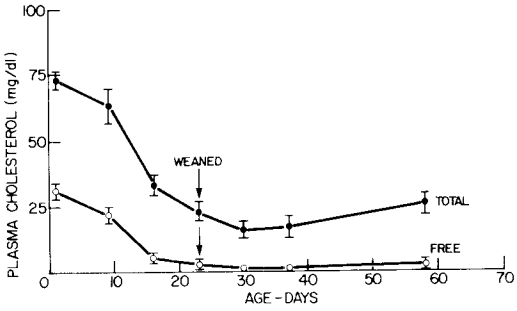

FIG. 4. Plasma cholesterol levels in guinea pigs. Results are given as Mean ± S.E.M. for five males and six females from three different litters. The values were pooled since there was no sex difference.

The puppies used in the first experiment (Fig. 2) weighed 432 ± 9 g at birth and gained 2460 ± 20 g from birth to 69 days of age. Weight gain was continuous except during the week after weaning when some of the puppies lost weight. Litters 1 and 2 (Fig. 3) weighed 445 ± 12 and 403 ± 7 g initially, and gained 3670 ± 215 and 2520 ± 295 g, respectively, between birth and 59 days of age. Weaning had little or no effect on the rate of weight gain of these puppies. From 59 to 100 days of age, the pups fed meat gained 1142 ± 329 g and those fed chow gained 931 ± 128 g, but this difference was not statistically significant.

Guinea Pigs

The results obtained with guinea pigs are shown in Figure 4. In this case, the first samples, taken when the animals were one day old, had the highest level of plasma cholesterol. Samples taken from three other animals within 3-4 hr of birth, gave values of 70, 73, and 83 mg/dl total cholesterol and 21, 22, and 25 mg/dl of free cholesterol. The plasma cholesterol decreased slowly during the first week of life and then more rapidly. It remained in the range of 25-30 mg/dl from the age of 3 wk until the experiment was terminated when the animals were 8 wk old.

DISCUSSION

The changes in plasma cholesterol in kittens and puppies followed a pattern similar to that observed previously in other species (1). Guinea pigs were different in that the highest plasma cholesterol levels were observed during the first day of life.

In all three species, as in others studied previously, weaning was accompanied by a decrease in plasma cholesterol levels. Sometimes the level began to drop prior to weaning, perhaps because the young, having access to the mother's feed, were often partially weaned before they were actually removed from the mother. Guinea pigs, in particular, begin to eat solid rations within a few days after birth, and this may explain the absence of any rise in plasma cholesterol during the suckling period.

The hypercholesterolemia in suckling animals appears to be due to ingested milk lipids (1), and the decrease at weaning may be a result of changing from a high fat to a low fat diet. Fat constitutes about 30% of the total solids of milk from cats and about 40% in the case of dogs and guinea pigs (7). The fatty acid composition of milk lipids may also influence plasma cholesterol levels during the suckling period. Milk fats of the guinea pig have been reported to contain 17% linoleic acid compared to 9% in those of the dog (8).

The plasma cholesterol levels observed in kittens, puppies, and guinea pigs during the early postweaning period in the present experiments are comparable to values recorded in the literature for these species (9-11). This is also generally true of other animal species investigated (12). This suggests that cholesterol levels tend to remain fairly constant in animals after the time of weaning, although the level has been reported to increase with age in rats (13) and in female Beagle dogs (14).

A secondary rise in plasma cholesterol postweaning was seen in all three litters of puppies in the present experiments (Figs. 2 and 3), but this appeared to be temporary in two litters followed to 100 days of age (Fig. 3). It is not certain whether this is a constant phenomenon with some physiological basis. In the experiments with puppies, the kind of diet fed after weaning (horse meat or dog chow) appeared to have little effect on the level of plasma cholesterol. In contrast, Goddard et al. (15) reported that young male beagles fed an all-meat diet had higher serum cholesterol levels than those fed a dry-type diet.

ACKNOWLEDGMENTS

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A Comparative in vivo Study of Intestinal Absorption of Biliary Phosphatidylcholines and Micellar Phosphatidylcholines in the Rat

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ABSTRACT

An in vivo study was performed using rats with the purpose of comparing the absorption of native biliary and purified phosphatidylcholines. The latter were purified from bile and solubilized in the form of mixed micelles of bile salts-phosphatidylcholines-cholesterol. The animals all bore bile duct diversions, and were divided into two groups: one had a normal pancreatic secretion while in the other group the pancreatic duct was ligated. Animals with normal pancreatic secretion showed comparable rates of absorption of micellar and biliary phosphatidylcholines. In the absence of normal pancreatic secretion, the rate of absorption of biliary phosphatidylcholines was unchanged, whereas that of micellar phosphatidylcholines markedly decreased. The results are consistent with the concept that some billary phosphatidylcholines are absorbed independently of pancreatic secretion in an unhydrolyzed form.

INTRODUCTION

Exogenous phosphatidylcholines are rapidly degraded in the intestinal lumen by pancreatic phospholipase A_2 prior to absorption (1-4). Purified phosphatidylcholines, which are solubilized by bile salts in the form of mixed micelles, are similarly degraded in vitro (5). Until recently, it was assumed that biliary phosphatidylcholines and exogenous phospholipids are processed similarly in the intestinal lumen (6,7). Evidence of a micellar-type organization of phosphatidylcholines in the bile (8) has reinforced this concept.

Nevertheless, recent work seems to weaken this hypothesis. Results obtained in our laboratory have shown that the biliary phosphatidylcholines are organized in a lipoproteic complex (9-11) and that they participate in the intraluminar digestion of fats in the rat (12-13). Furthermore, rat biliary phosphatidylcholines are apparently highly resistant to attack by pancreatic phospholipase A_2, both in vivo and in vitro (14), and would thus be absorbed largely intact (15).

The results presented in the present report show that the intestinal absorption of native biliary phosphatidylcholines is different from that of phosphatidylcholines purified from bile and then solubilized by bile salts in vitro forming mixed micelles.

MATERIALS AND METHODS

Animal Experimentation

Male Wistar rats (about 200 g) were fasted for 24 hr preceding the operation. They were then divided into two groups.

Group A: The animals were anaesthetized with ether, the abdominal cavity was opened and a derivation was effected by introducing a type PE 10 catheter in the proximal portion of the bile duct. The bile, recovered on ice, was used within 24 hr. A second PE 10 catheter was introduced distal to the first, before the entry of the pancreatic duct, in order to permit injection into the duodenum without disturbing pancreatic secretion.

Group B: The derivation of the bile duct was performed as in group A. The pancreatic duct was ligatured near its distal end and a second PE 10 catheter was introduced distal to the first, feeding directly into the duodenum. Thus, pancreatic secretion was prevented in this group. These surgical procedures have the advantage of leaving the stomach and duodenum intact.

After the surgical intervention, the animals were placed in restraining cages. Intestinal perfusions of 5% glucose, 0.5% NaCl, 0.05% KCl (3) were administered into the distal catheter at a flow-rate of 1 ml/hr.

Experimental manipulations were performed 24 hr after the operation. The animals received, for a short time, either bile or phosphatidylcholines purified from bile and solubilized in vitro in the form of mixed micelles of bile salts-phosphatidylcholine-cholesterol. The proportions of the various constituents were the same as in rat bile. The glucose-saline perfusion was then re-established for at a rate of 1 ml/hr.

The small intestine was ligatured both at the level of the pylorus and just proximal to the cecum, and its content withdrawn immediately and placed in ice. To minimize loss of phosphatidylcholines which could be adsorbed to the intestinal mucosa, the intestine was rinsed twice with glucose-saline solution. It was verified that under these experimental conditions, the injected solutions did not reach the cecum since the distal small intestine was empty. Moreover, recent results have shown that the site of biliary phosphatidylcholine absorption is in the proximal small intestine (16). For these reasons, we feel that almost all of the infused phosphatidylcholines which could be recovered had been absorbed.

To verify that the capacity of intestinal absorption was maintained, we monitored the phosphatidylcholine content of bile after infusion of the various solutions. We observed an increase in the phosphatidylcholine secretion indicating an intact enterohepatic circulation of bile salts (11). Furthermore, Sklan et al. (18) have concluded that in rat, the proximal small intestine is as important in bile salt absorption as the distal small intestine.

Identification and Determination of Phospholipids

A volume of intestinal content was mixed with 25 volumes of chloroform:methanol 2:1 v/v in such a way as to obtain one phase. The mixture was filtered through glass wool and evaporated under nitrogen at 37 C. The residue containing phospholipids was taken up in chloroform:methanol 90:10 v/v and separated on thin layer of silica gel (Schleicher and Schüll thin layer plates F 1500 on plastic supports). The plates had been soaked first in 2N $HClO_4$ for 24 hr and then rinsed with water in order to eliminate contaminating inorganic phosphorus. Chromatograms were developed with chloroform:methanol:20% NH_4OH 70:30:5 v/v (19). Spots corresponding to phosphatidylcholines and lysophosphatidylcholines were revealed with molybdenum blue (20), and it was verified that this compound did not affect the subsequent colorimetric analysis. The spots were scraped and phosphorus content determined after mineralization with $HClO_4$ (21). Blanks were systematically included and were very weak. This reliable method thus permits the calculation of unabsorbed lipid phosphorus in the intestinal luminal contents.

The amount of absorption is the difference between quantity injected and the amount retrieved in the intestinal contents.

Preparation of Micellar Phosphatidylcholines

Sodium taurocholate (Calbiochem A grade) and cholesterol (Steraloïd) were used without further purification. Phosphatidylcholines were purified from rat bile by chromatography on silicic acid (22) and were solubilized in vitro in the form of mixed micelles of bile salts-phosphatidylcholines-cholesterol in the same proportions as found in native rat bile (ca. 35 μmole of taurocholate, 2.5 μmol cholesterol, and 5 μmol phosphatidylcholines per ml).

RESULTS AND DISCUSSION

The quantity of biliary phosphatidylcholines injected was similar to that recovered in the bile after one hour. The 1-hr incubation time was used because we observed that no significant amount of phosphatidylcholine was recovered after longer incubation times and that the infusate did not reach the cecum after 1 hr. For comparison, equivalent amounts of micellar phosphatidylcholines were infused for 1 hr.

It has been shown that there is an almost total absence of phospholipids in the intestinal content if biliary-diverted rats are starved for 24 hr and then receive an intraduodenal injection of 10 mM bile salts. Thus, it can be concluded that the phospholipids recovered in the intestinal content were derived from infused solutions (13).

Group A animals–Biliary derivation with retention of pancreatic secretion (Table IA): The results obtained for these animals show that the absorption of both biliary and micellar phosphatidylcholines is comparable. A significant absorption of phospholipids is seen, since total unabsorbed lipidic phosphorus does not exceed 22% of the injected material.

Group B animals–Biliary derivation with suppression of pancreatic secretion (Table IB): In comparison with group A animals, the absorption of native biliary phosphatidylcholines was not significantly changed. The absorption of micellar phosphatydylcholines was markedly decreased, since an average of 83% of injected phospholipid was recovered in the intestinal luminal contents.

In the presence of normal pancreatic secretion, the absorption of micellar phosphatidylcholines (mixed micelles of bile salts-phosphatidylcholines-cholesterol) is quite significant, being in the order of 85% of the input. In contrast, the suppression of pancreatic secretion leads to a significant decrease in absorption of phosphatidylcholines. In the latter case, ca. 83% of injected lipid phosphorus was not absorbed. These results agree with those obtained previously which showed that exogenous phosphatidylcholines must first be degraded by pancreatic phospholipase A_2 before being

TABLE I

Analyses of Lipid Phosphorus in Intestinal Content of Rats
One Hour after Intraduodenal Injection of Phosphatidylcholines

Injected phosphatidyl-choline (mg)	Unabsorbed phosphatidyl-choline (mg)	Unabsorbed lysophosphatidyl-choline (mg)	% of absorbed lipid phosphorus
A. Biliary diversion with preservation of pancreatic secretion (Group A)			
	Bile phosphatidylcholines		
8.01	0.80	0.56	83.0
8.13	0.25	0.30	93.3
8.13	0.13	0.03	98.1
8.25	0.80	0.85	80.0
	Micellar phosphatidylcholines		
8.01	0.18	0.37	93.2
7.91	1.21	0.52	78.2
7.91	0.79	0.39	85.1
8.23	0.49	0.57	87.2
B. Biliary diversion with suppression of pancreatic secretion (Group B)			
	Bile phosphatidylcholines		
7.85	0.18	0.20	85.2
7.85	0.39	0.34	90.7
8.13	1.21	1.38	68.2
8.13	0.93	0.85	78.2
	Micellar phosphatidylcholines		
7.90	5.05	2.60	3.2
8.37	4.43	1.84	15.1
8.17	2.77	2.45	36.0
7.85	5.34	2.35	2.1

absorbed by the intestinal mucosa.

When micellar phosphatidylcholines are injected into animals whose pancreatic secretion had been suppressed, appreciable quantities of nonabsorbed lysophosphatidylcholines were recovered in the intestine, indicating that significant phospholipase activity persists. This activity is difficult to totally eliminate and could either have a pancreatic origin or reflect cell renewal of the intestinal mucosa. The first possibility was verified to some extent since several precipitation bands were obtained between rat pancreatic juice antiserum and intestinal contents 24 hr after pancreatic bile duct derivation. It is possible that certain pancreatic enzymes remain adsorbed onto the mucosa (24) or that some pancreatic secretion persists despite ligature of the pancreatic duct. It must be pointed out that although an appreciable hydrolysis of micellar phosphatidylcholines occured, the absorption of lysophosphatidylcholine was impaired considerably. We do not have an explanation for this observation.

It can be imagined that suppression of pancreatic secretion does not affect the absorption of billary phosphatidylcholines if they are highly resistant to pancreatic phospholipase A_2 (13,14). In the presence of pancreatic secretion, both biliary and micellar phosphatidylcholines are absorbed to a comparable extent. These results are similar to those of Arnesjo et al. (7). Thus, it appears that no difference in absorption of the two types of phospholipids occurs when pancreatic secretion is normal. The suppression of pancreatic secretion, however, leads to a sharp discrimination between the amount of absorption of native and micellar phosphatidylcholines since the absorption of the former is unchanged from that observed under conditions of normal pancreatic function. In view of the results we obtained in the absence of pancreatic secretion and contrary to the conclusion of Arnesjo et al. (7), an identical mode of absorption of native and micellar phosphatidylcholines under physiological conditions cannot be concluded.

The difference in absorption, in the absence of pancreatic secretion, would suggest different modes of absorption for the two types of phosphatidylcholines. Indeed, the work of Boucrot strongly suggests that native phosphatidylcholines are largely absorbed in an unhydrolyzed

state (15). Since the resistance to pancreatic phospholipase A_2 is not total, it is possible that a small proportion (15%) of biliary phosphatidylcholines have a fate similar to that of the exogenous lipids (13-15).

The difference in absorption of the two phospholipids could be due to physicochemical differences. Our work has shown that a lipoprotien complex exists in bile appearing as a strong association between phosphatidylcholines and a polypeptide fraction (11). In this configuration, the fatty acid chain could be protected from hydrolysis by phospholipase A_2. In fact, it is known that a part of the enzyme must first undergo a penetration step before hydrolysis can occur (25).

It has been suggested by Rampone (26,27) and O'Doherty et al. (28) that phosphatidylcholines are important in the intestinal absorption of fat, and our previous results (12,13) show a participation of biliary phosphatidylcholines in intraluminal triglyceride lipolysis. The limited data presented in this paper are in accordance with the results obtained by Boucrot and Clement (14,15) and are consistent with the idea that a major part of biliary phosphatidylcholines are absorbed independently of pancreatic secretion in an unhydrolyzed form. These results emphasize the importance of further research in this area. It is our aim to clarify the exact mechanism of biliary phosphatidylcholine transport across the intestinal mucosa.

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Hypertriglyceridemia in Ehrlich Ascites Carcinomatous Mice: Tumor and Mouse Strain Differences[1,2]

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ABSTRACT

Ehrlich ascites carcinoma growth in mice induces hypertriglyceridemia. The degree of hypertriglyceridemia found in one laboratory (Spector's) was much greater than we observed in our laboratory. Moreover, major differences were reported with respect to fasting (no effect on tumor extracellular fluid triglyceride levels in Spector's tumor-bearing mice; marked decrease in ours). We have obtained tumorous CBA mice from Spector's laboratory and have studied them simultaneously with our Swiss-Webster mice. Triglyceride levels of the above two groups and from two controlled cross-over groups, included to evaluate the influence of mouse and tumor strains on hypertriglyceridemia, were determined. The CBA mice had intense hypertriglyceridemia and high triglyceride levels in tumor extracellular fluid regardless of the subline source of ascites tumor. On the other hand, only mild hyperlipidemia was induced with both strains of tumor in Swiss-Webster mice. Thus, the variations in plasma and tumor extracellular fluid triglyceride levels probably arise from the mouse strains and not from variations in the tumor subline. Fasting caused a decrease in both plasma and tumor extracellular fluid triglyceride concentrations in CBA, as well as in Swiss-Webster mice. A mouse strain difference was also evident from a significant decrease in wet weights of adipose tissues like epididymal fat, inguinal fat, and intermuscular fat with tumor growth in the CBA strain which was not observed in the Swiss-Webster strain at the corresponding stage of tumor growth. Study of these strain differences may lead to an understanding of factors that regulate hyperlipidemia.

INTRODUCTION

Our laboratory has been studying the nature and origin of lipids needed by tumor cells for their growth and metabolism. One possible source of tumore lipids is the tumor extracellular fluid free fatty acids (FFA), the importance of which has been stressed by Spector (1). Studies, both in vitro and in vivo, have shown that there is a very rapid exchange of FFA between the tumor extracellular fluid and the Ehrlich ascites carcinoma cell (2,3). However, Mermier and Baker (4) found that there was very little transport of FFA from plasma to the tumor extracellular fluid (or vice versa); they suggested that the tumor fluid may derive its FFA from the neighboring host tissues by direct transport not involving blood plasma. Brenneman et al. (5), working with CBA mice, have presented evidence for yet another source of lipid for tumor; namely, triglyceride fatty acids. They found high levels of triglycerides in the tumor extracellular fluid associated with pronounced hyperlipidemia in blood plasma of CBA mice bearing Ehrlich ascites tumor. We have recently shown that Ehrlich ascites carcinoma induces hyperlipidemia of a milder degree and the plasma and tumor extracellular fluid triglyceride levels are strikingly less in Swiss-Webster mice than in CBA mice (6). It was, therefore, of interest to study the factors that regulate the levels of triglyceride in plasma in the two strains of mice, especially since Brenneman et al. (6) have hypothesized that tumor lipids may be derived from plasma very low density lipoprotein (VLDL) triglycerides. The present investigation was undertaken to determine whether we could confirm, under controlled conditions, that CBA mice with Spector's subline of tumor had a different hypertriglyceridemic response than Swiss-Webster mice with our tumor, and, if so, to establish whether the difference was due to variation in the mouse and/or the tumor strain. At the same time, possible strain differences in the response of the host's fat depots to tumor growth were explored.

EXPERIMENTAL PROCEDURES

Tumors

In Experiment I, two sublines of tumors

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TABLE I

Triglyceride Concentrations in Blood Plasma and Tumor Extracellular Fluid in Two Mouse Strains and Tumor Sublines and Their Crossover Combinations[a]

		Triglyceride mg/dl		
		Tumorous mice		Controls
Mouse strain	Tumor subline	Blood plasma	Extracellular fluid	Blood plasma
Swiss-Webster	McKee's (our)[b]	449 ± 63	50 ± 9	169 ± 17
CBA[d]	Spector's[c]	1032 ± 64	309 ± 72	264 ± 40
Swiss-Webster	Spector's[c]	453 ± 149	27 ± 9	96 ± 16
CBA[d]	McKee's (our)[b]	1493 ± 114	346 ± 52	192 ± 37

[a]Mean ± S.E. from 5-6 mice per group.

[b]The inoculum and tumor age were 15 x 10^6 cells/mouse and 8 days, respectively.

[c]The inoculum and tumor age were 3 x 10^6 cells/mouse and 10 days, respectively.

[d]Blood plasma and tumor extracellular fluid triglyceride concentrations in CBA mice differed from the corresponding Swiss-Webster values significantly ($P<0.01$).

were studied in two strains of mice. The first subline of tumor, one that has been serially transplanted in our laboratory for many years, was the Ehrlich Lettré hyperdiploid carcinoma, originally obtained from Dr. R. McKee (Biological Chemistry Department, UCLA School of Medicine, Los Angeles, CA). It was transplanted (4) in 6-10 wk old, male, Swiss-Webster mice (Hilltop Animals, Inc., Chatsworth, CA) weighing 32-38 g. The inoculum of washed tumor cells was 15 x 10^6 cells/mouse injected intraperitoneally. The tumor size was at its maximum on day 14. The mean survival time of tumor-bearing mice was 15 days. Another subline of tumor was obtained from Dr. A. Spector (University of Iowa, Iowa City, IA) and was transplanted into male CBA mice (Cumberland Farms, Clinton, TN), 6-10 wk old, weighing 18-22 g. This subline was obtained in 1963 from Dr. M.W. Woods, National Cancer Institute, National Institutes of Health, Bethesda, MD, who had maintained it in C_3H mice; Spector and coworkers have carried it since then in male CBA mice (5). The inoculum of washed tumor cells was the same as used by Brenneman et al. (5) which is 3 x 10^6 cells/mouse. Under these conditions, the mean survival time of the cancerous mice was 15-16 days. In Experiment II, both Swiss-Webster mice and CBA mice were inoculated with 15 x 10^6 cells/mouse of McKee's tumor and the induction of hypertriglyceridemia in the two strains of mice was followed by means of blood and tumor sampling (see below) every alternate day.

In cross-over experiments (Experiment I), the inoculum was 15 x 10^6 cells/mouse (McKee's tumor) and 3 x 10^6 cells/mouse (Spector's tumor); the two cross-over groups were: Swiss-Webster mice inoculated with 3 x 10^6 cells of Spector's Ehrlich ascites tumor (EAT) and CBA mice inoculated with 15 x 10^6 cells of McKee's tumor.

Both control and tumorous mice were housed in community cages (6/cage) and were allowed to eat Purina chow diet ad libitum. Water was also available ad libitum. Body weights were recorded every other day to monitor tumor growth.

Sampling

Blood was obtained from the opthalmic venous sinus (7) in heparinized capillaries around 9 AM. The blood was immediately centrifuged at 1000 rpm for 1 min at 4 C and the plasma separated from the red cells. Tumor samples were obtained by puncturing the abdomen of tumor-bearing animals with a needle. The needle was removed from the peritoneal cavity and ca. 0.1-0.2 ml tumor was allowed to drip into heparinized capillaries by gently squeezing the abdomen. Extracellular fluid was separated from the tumor cells by centrifugation as described above.

To study the effect of fasting, both Swiss-Webster and CBA mice (6/group) were fasted 24 hr from days 7 and 9, respectively, and sampling of blood and tumor extracellular fluid was done on days 8 and 10 as described above. In a separate study of control and tumorous CBA mice, the duration of fasting was reduced to 16 hr (5), beginning on the seventh day of tumor growth.

Isolation of Tissues

Fat tissues were isolated from control and tumorous mice (Swiss-Webster mice with McKee's tumor and CBA mice with Spector's tumor; inoculum as in Table I). Epididymal fat pad, intermuscular fat of the popliteal region

TABLE II

Effect of Fasting on the Blood Plasma and Tumor Extracellular Fluid in Swiss-Webster and CBA Mice Bearing Ehrlich Ascites Tumor[a]

Study	Mouse strain	Tissue fluid	Dietary state	Triglyceride (mg/dl) Controls[b]	Triglyceride (mg/dl) Tumorous mice[b]
I	Swiss-Webster	Blood plasma	Fed	169 ± 17 (6)	449 ± 63 (6)
		Blood plasma	24-hr fasted	119 ± 17 (4)[c]	67 ± 18 (4)[d]
	CBA	Blood plasma	Fed	250 ± 35 (5)	720 ± 73 (5)
		Blood plasma	24-hr fasted	146 ± 18 (5)	186 ± 20 (5)[d]
II	CBA	Blood plasma	Fed	284 ± 25 (6)	600 ± 16 (6)
		Blood plasma	16-hr fasted	174 ± 9 (6)[d]	272 ± 33 (6)[d]
		Tumor extracellular fluid	Fed	–	155 ± 47 (6)
		Tumor extracellular fluid	16-hr fasted	–	49 ± 9 (6)[d]

[a]Tumor age and inoculum were the same as in Table I, except CBA mice in Study II received 15 x 10^6 cells/mouse of McKee's tumor. Triglyceride determinations were made on Day 8 in Study II.

[b]Mean ±S.E. Number of mice in each group is given in parentheses.

[c]Not significantly different as compared to the fed-control group.

[d]Significantly ($P<0.01$) different as compared to the fed-control or-tumor group.

[e]Significantly ($P< 0.05$) different as compared to the fed-tumor group.

(8), and inguinal fat (9) were dissected out from fed and 24-hr-fasted control and tumorous mice immediately upon sacrifice. In cases where the fat pads had extraneous fluid surrounding them, this fluid was absorbed by cutting the pads into small pieces and gently squeezing them with a filter paper before the tissue weights were recorded.

Analysis of Triglycerides

Triglycerides of blood plasma and extracellular fluid were estimated using the method of Galletti (10) with slight modifications; 20 µl plasma and 20 µl saline were taken for analysis instead of 40 µl plasma described in the original method. Suitable isopropyl ether aliquots containing neutral lipids were used depending upon the expected triglyceride level in the sample. Plasma and extracellular triglyceride values were read from a simultaneously run tripalmitin standard curve.

RESULTS

Experiment I: Simultaneous Repetition of Earlier Experiments (5,6) in Two Mouse Strains

The conditions used in this initial study were based on those used earlier in the two laboratories in which discrepant observations were reported (5,6). As a result, two different tumor cell inocula were used (see Methods), and the triglyceride concentrations were determined on the eighth and tenth days of tumor growth in Swiss-Webster (6) and CBA mice (5), respectively, the times that have been reported for development of maximal hypertriglyceridemia. Triglyceride levels of blood plasma and tumor extracellular fluid in Experiment I are shown in Table I. The results obtained for Swiss-Webster mice in this study differed from those in our previous work (6). The ascites fluid triglyceride concentrations were double those previously reported and blood plasma triglycerides were also elevated considerably. Still, the blood plasma triglycerides in tumorous CBA mice were significantly higher than those in Swiss-Webster mice (1032 mg/dl vs. 449 mg/dl). More striking was the six-fold higher triglyceride concentration in the ascites fluid of CBA mice as compared to Swiss-Webster mice. Thus, our results confirm the pronounced hypertriglyceridemia and a sizable triglyceride pool in tumor extracellular fluid in CBA mice bearing Ehrlich ascites tumor, first reported by Brenneman et al. (5); these data (Table I) also rule out the possibility that methodological differences are responsible for the conflicting findings from Spector's laboratory and our laboratory.

Results of studies to find out whether the changes in degrees of hyperlipidemia arose from differences in mouse strains or from the tumor sublines are also tabulated in Table I. The tumor inoculum (15 x 10^6 cells/mouse of McKee's tumor and 3 x 10^6 cells/mouse of Spector's tumor) was kept the same in the crossover studies to obtain maximal hyperlipidemia with tumor ages of 8 days and 10 days. The crossover combinations were produced by transplanting tumor cells obtained from Spector's laboratory to Swiss-Webster mice and our subline of tumor to CBA mice. As is shown in Table I, ascites tumor induced pronounced hyperlipidemia (1032 mg/dl and

TABLE III

Triglyceride Concentrations of Blood Plasma and Tumor Extracellular Fluid in Swiss-Webster and CBA Mice Bearing Ehrlich Ascites Tumor[a]

Mouse strain	Day of tumor	Triglycerides (mg/dl) Controls, blood plasma[b]	Tumorous mice, blood plasma[b]	Tumorous mice, extracellular fluid[b]
CBA	4	276 ± 25	134 ± 8[c]	–
	6	341 ± 31	626 ± 40[c]	126 ± 11
	8	284 ± 25	600 ± 116[d]	155 ± 41
	10	274 ± 29	864 ± 188 (7)[c]	158 ± 39 (7)
Swiss-Webster	4	189 ± 40	102 ± 46 (4)[e]	–
	6	180 ± 54	350 ± 82 (4)[e]	47 ± 6 (4)
	8	159 ± 20	195 ± 30 (4)[e]	46 ± 4 (4)
	10	132 ± 10	149 ± 30(3)[e]	32 ± 11 (3)

[a]Tumor inoculum was 15 x 10^6 cells/mouse of McKee's tumor.

[b]Mean ± S.E. from 8 animals from each group unless otherwise marked in parentheses.

[c]Significantly ($P<0.01$) different as compared to the control, blood plasma group.

[d]Significantly ($P<0.02$) different as compared to the controls, blood plasma group.

[e]Not significantly different as compared to the controls, blood plasma group.

1493 mg/dl) in CBA mice, irrespective of the tumor subline. The Swiss-Webster mice, on the other hand, developed only moderate hyperlipidemia (449 mg/dl and 453 mg/dl) with both tumor sublines. Tumor extracellular triglyceride levels were also much higher in CBA mice than in Swiss-Webster mice. Thus, the variations in degrees of hyperlipidemia probably resulted from the differences in the mouse strains rather than in the tumor sublines. Although a highly significant difference between mouse strains was clearly established in these studies, we have been unable to control the degree of maximal cancer-induced hypertriglyceridemia in either strain. Thus, peak plasma triglycerides varied in different experiments from 192 to 449 mg/dl (mean) in tumorous Swiss-Webster mice and from 700-1600 mg/dl (mean) in cancerous CBA mice. Similar variations in the degree of cancer-induced hypertriglyceridemia have been previously reported by Brenneman et al. (5) in the CBA mice. In all cases, mice were fed ad libitum; hence, we do not know when the mice ingested food prior to blood sampling. Since fasting, as shown earlier and confirmed here (see below), lowers plasma triglycerides in cancerous mice, the variations in degree of hyperlipidemia from one experiment to another may reflect, in part, inadequate control of the animals' dietary state.

The effect of fasting on plasma triglycerides of normal and tumorous mice of the two strains is given in Table II. There was a significant drop in triglyceride levels in 24-hr fasted, tumorous Swiss-Webster mice. We have shown earlier that the fall in plasma triglycerides, associated with a pronounced decrease in levels of tumor extracellular fluid triglycerides, occurs after a 24-hr fast (6). However, Brenneman et al. have reported no fall in tumor extracellular fluid ("plasma") triglycerides after EAT-bearing, CBA mice were fasted for 16 hr (5). The data shown in Table II are in direct contrast to the findings of Brenneman et al. (5). A significant decrease in blood plasma triglyceride levels occurred in cancerous CBA mice after a 24-hr fast (720 to 186 mg/dl), and reducing the fasting period to only 16 hr produced a similar decrease (600 to 272 mg/dl). This drop in plasma triglycerides was associated with a comparable fall in tumor extracellular fluid triglyceride levels in the 16-hr-fasted CBA mice (155 to 49 mg/dl).

Experiment II; Constant Donor Inoculum and Varying Mouse Strains

In Experiment I, comparisons in degrees of hyperlipidemia in the two mouse strains and their cross over combinations were made on days 8 and 10, allowing for a probable shift in hyperlipidemia with a lowering of tumor inoculum from 15 x 10^6 cells to 3 x 10^6 cells/mouse (5,6). To confirm the validity of this selection of peak days, a choice that was made on the basis of earlier published reports, a controlled, serial study of hyperlipidemia in tumorous Swiss-Webster and CBA mice was carried out using a constant inoculum of 15 x 10^6 cells/mouse of McKee's tumor (Experiment II). As shown in Table III, triglyceride concentrations increased with tumor growth in both groups of mice; however, the CBA mice clearly developed a more intense hyper-

TABLE IV

Effect of Tumor Growth and Fasting on Fat Depots in CBA and Swiss-Webster Mice[a]

Mouse strain	Dietary state	Group	Wet tissue weight per fat pad (mg)		
			Epididymal fat	Popliteal fat (intermuscular fat)	Inguinal fat
CBA	Fed	Control (4)	151 ± 13	9.6 ± 0.3	148 ± 5
		Tumor (4)	89 ± 11[b]	1.0 ± 0.3[c]	30 ± 9[c]
	24-hr fasted	Control (4)	108 ± 5	6.8 ± 0.9	92 ± 13
		Tumor (6)	16 ± 6[c]	Trace	Trace
Swiss-Webster	24-hr fasted	Control (3)	240 ± 41	8.4 ± 1.8	99 ± 8
		Tumor (3)	422 ± 5[b]	6.7 ± 1.8[d]	112 ± 6[d]

[a]Mean ± S.E. Number of mice per group is given in parentheses.

[b]Significantly ($P<0.05$) different when compared to the control group.

[c]Significantly ($P<0.001$) different when compared to the control group.

[d]Not significantly different when compared to the control group.

lipidemia than the Swiss-Webster mice between the 8th and 10th days of tumor growth. The ascites fluid triglyceride concentrations were also significantly higher in cancerous CBA than in Swiss-Webster mice. Thus, the differences observed between Swiss-Webster and CBA mice with respect to EAT-induced hypertriglyceridemia are not simply the result of varying inoculum sizes. Increasing the inoculum from 3 x 10^6 to 15 x 10^6 cells only seems to shift the tumor growth curve and the time of maximal hyperlipidemia by 2-3 days as one would expect from a 24-hr doubling time during the log phase of tumor growth. The time of maximal hypertriglyceridemia in the CBA mice was ill-defined in Experiment II; however, additional studies indicate that the high values on day 10 may reflect the development of a secondary maximum. Such secondary peaks have been observed in individual mice of both strains (unpublished observations). This phenomenon is currently being investigated.

In order to study the effect of tumor growth on fat depots of the host, three discrete fat bodies were isolated from control and tumorous mice of the two strains. The animals used for tissue isolation were the same as in the cross-over studies given in Table I. A striking difference between the wet tissue weights of three fat depots of tumorous CBA mice and the control nontumorous mice was found, as illustrated in Table IV. There was a significant depletion of epididymal adipose tissue with tumor growth in the CBA mice as previously observed by Spector (personal communication). Intermuscular fat and inguinal fat were also significantly reduced in the tumorous CBA mice. This reduction in wet tissue weights was more drastic in 24-hr-fasted CBA mice, in which the inguinal and popliteal fat depots disappeared completely. A different phenomenon occurred in Swiss-Webster mice bearing EAT (TAble IV). There was no change in wet tissue weights of distal fat stores such as the intermuscular fat or inguinal fat in fasted tumorous mice as compared to the controls. In the epididymal fat pad there was, in fact, an increase in wet tissue weight in cancerous mice which appeared to result from infiltration of the adipose tissue by the surrounding tumor cells.

DISCUSSION

The present study, using tumorous CBA mice obtained from Spector's laboratory, confirms their earlier report of pronounced hypertriglyceridemia in these animals (5). At the same time, we have confirmed our own earlier work (6) showing that a less-pronounced hyperlipemia develops in our Swiss-Webster mice during growth of Ehrlich ascites carcinoma. By carrying out these initial studies in one laboratory, we have been able to establish, first of all, that the difference in degree of hyperlipemic response to tumor growth results from a variation either in the mouse strain or in the tumor subline (11,12), rather than from some interlaboratory technical variable. Then, by conducting crossover studies, we were able to establish that the difference in hyperlipemic response is not due to variation in tumor subline; rather, it is due to some unknown metabolic difference between CBA and Swiss-Webster mice. This difference in mouse strains was clearly evident in each experiment despite the marked and poorly understood variation in degree of hypertriglyceridemia that we observed from one experiment to another. If one could elucidate the nature of this strain difference, as well as the causes of this variability,

one might also obtain important information regarding the molecular basis of cancer-induced hypertriglyceridemia.

The difference in the two strains of mice was not restricted to degrees of hyperlipemia. Comparable tumor growth caused the disappearance of fat stores from one strain (CBA), but not the other (Swiss-Webster). This suggests that tumor growth alters the balance between lipid deposition and lipid mobilization (lipolysis) differently in the two mouse strains. It will be of great interest to explore this phenomenon and to determine the relationship, if any, of the variations in hyperlipemic response to tumor growth.

The contribution made by VLDL-triglyceride fatty acids to the rapidly turning-over FFA pool of the Ehrlich ascites carcinoma's extracellular fluid in these two strains of mice is still unknown. It is now established that VLDL-triglycerides in the tumor extracellular fluid of fed mice of either strain are present in much higher concentration than FFA. The fact that Mermier and Baker failed to detect high concentrations of tumor fluid triglycerides (4) probably indicates that their cancerous animals had not eaten prior to the study; fasted, Swiss-Webster mice usually have very low levels of tumor fluid triglycerides (6). Whether the extracellular triglycerides are a significant source of fatty acids for the tumor's growth and energy needs, as Brenneman et al. have suggested (5), remains to be established. Preliminary experiments in our laboratory using both fed and fasted Swiss-Webster mice suggest that the transport of labeled VLDL-triglyceride fatty acids from plasma to Ehrlich ascites carcinoma is extremely slow (almost negligible compared to uptake by the host's tissues; unpublished observations).

Until now, it has not been clear whether or not food intake influenced hypertrilyceridemia in cancerous rodents. Posner has reported that the hypertriglyceridemia of Walker 256-carcinomatous rats disappeared when fat absorption was interrupted (13); he concluded that the elevated blood lipids were derived from exogenous fat. However, based upon their failure to observe a reduction in tumor fluid triglyceride levels after dietary fat was excluded, by fasting the cancerous mice, Brenneman et al. concluded that the hyperlipemia of Ehrlich ascites carcinomatous mice is probably of endogenous origin (5). In this regard, our results are not in accord with those from Spector's laboratory. We have previously shown that fasting of cancerous, Swiss-Webster mice causes a pronounced fall in both plasma and extracellular fluid triglyceride levels (6). In the present study, we had the opportunity to re-examine this question, using both strains of mice. Our results clearly establish that a marked reduction in plasma and tumor fluid triglycerides occurs during fasting in both strains of tumorous mice. Based upon these data, the possibility that dietary lipid may contribute to hypertriglyceridemia cannot be ruled out. Studies designed to resolve this controversy are in progress (14).

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Changes in the Acyl and Alkenyl Group Composition of Cardiac Phospholipids in Boars Fed Corn Oil or Rapeseed Oil[1]

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ABSTRACT

Boars fed diets containing rapeseed oil for 8 weeks showed significantly higher levels of neutral lipids and similar levels of phospholipids, compared to those fed corn oil. Erucic and eicosenoic acids were found to be high in ethanolamine phosphoglycerides, and in particular alkenyl acyl-ethanolamine phosphoglyceride. Furthermore, both long chain monoenes were incorporated preferentially in position 2 of the choline and ethanolamine phosphoglycerides. The alkenyl group composition of the cardiac lipids of pigs was influenced by dietary fatty acids. When rapeseed oil was fed, small amounts of 20:1 and 22:1 alkenyl constituents were detected.

INTRODUCTION

Several workers have demonstrated that inclusion of rapeseed oil (RSO) high in erucic (*cis*-13-docosenoic) acid in the diets of rats (1-3) and pigs (4-6) resulted in dramatic changes in the fatty acid composition of total cardiac lipids. Changes have also been reported in lipid classes of rat heart (7). In the present communication, a more detailed study is presented of the cardiac polar lipids of pigs fed rapeseed oil from a previous experiment (6) with specific reference to changes in the fatty acid and alkenyl ether composition of the choline and ethanolamine phosphatides.

TABLE I

Relative Concentration of Phospholipids in Cardiac Lipids of Boars Fed Experimental Diets for 8 Weeks

Phospholipid[a]	Diet	
	Corn	RSO[b]
Cardiolipin	8.8 ± 2.2[c]	10.3 ± 1.4
EPG	10.7 ± 2.5	15.9 ± 1.1*
SPG and IPG	5.1 ± 1.6	6.3 ± 0.2
CPG	44.8 ± 1.9	39.3 ± 2.3*
Sphingomyelin	24.3 ± 2.2	24.8 ± 1.5
2-acyl-CPG	6.5 ± 3.1	3.5 ± 1.2

[a]EPG = ethanolamine phosphoglyceride; SPG = serine phosphoglyceride; IPG = inositol phosphoglyceride; CPG = choline phosphoglyceride.

[b]RSO = a seed mixture of *Brassica campestris* var. 'Arlo' (15%) and 'Echo' (85%) containing 12.3% 20:1 and 22.3% 22:1.

[c]Values are mean ± SEM of 3 boars per diet; significant difference between diets at the 5% level (*).

MATERIALS AND METHODS

Yorkshire male pigs (boars), 9 to 10 wk of age, were fed a basl diet supplemented with 20% by weight of either corn oil or rapeseed oil as described previously (6). Three boars from each of the two dietary groups were killed on day zero, and successively after 1, 2, 3, and 8 wk of ad libitum feeding.

Hearts were homogenized and total lipids extracted with $CHCl_3$:MeOH (2:1) according to Christiansen (8). Total lipids were fractionated into neutral and polar lipid fractions by column chromatography on acid-treated Florisil (9). Polar lipids were further fractionated by thin layer chromatography (TLC) using the solvent $CHCl_3$:MeOH:H_2O 65:25:4, and bands were detected under UV light after spraying with a solution of Rhodamine B in methanol. Methyl heptadecanoate was added to all lipid classes to permit quantitation by gas liquid chromatography (GLC).

Transesterification was carried out by reacting the sample for 1 hr at 90 C with anhydrous HCl:MeOH (5% by wt). Methyl esters and dimethyl acetals were separated by TLC using 1,2-dichloroethane as developing solvent (10). Dimethyl acetals were converted to cyclic acetals of 1,3-propanediol (11) before analyses by GLC. Authentic cyclic acetals were prepared from authentic methylwesters (Nu Chek Prep., Elysian, MN); methyl esters were reduced to alcohols with $LiAlH_4$ in anydrous diethyl ether, oxidized to aldehydes with chromium tioxide-pyridine complex in methylene chloride (12), and acetylated with 1,3-propanediol and *p*-toluenesulfonic acid (11) in benzene.

A Hewlett-Packard Model 5830A GLC was used, equipped with flame ionization detectors and a digital integrator. Glass columns (1.8 m x 2 mm) were packed with 5% butanediol succinate on 80/100 mesh Chromosorb G (High Performance) or 10% SP-222-PS (Supelco Inc., Bellefonte, PA). Peaks were identified by co-

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TABLE II

The Fatty Acid Composition of Major Cardiac Lipid Classes of Boars Fed Experimental Diets for 8 Weeks[a]

Fatty acid[b]	TG		CL		CER		EPG		CPG		SPH	
	Corn	RSO	Corn	RSO	Corn	RSO	Corn	RSO	Corn	RSO	Corn	RSO
14:0	0.1[c]	0.1	0.6	0.4	0.9	0.8	0.2	0.5	0.4	0.4	0.7	1.5
16:0 DMA[d]	-	-	-	-	-	-	3.1	2.1	15.6	7.6**	-	-
16:0	19.6	16.3	5.0	6.1	8.1	5.3*	8.0	9.7	13.7	21.6**	16.5	18.5
18:0 DMA	-	-	-	-	-	-	11.0	5.1**	7.5	2.7*	-	-
18:0	21.3	19.4	6.0	10.8	12.1	9.5	32.3	31.2	22.2	19.5	16.9	13.8
18:1	15.1	22.8**	13.6	21.2**	11.7	18.5*	9.5	9.4	12.4	20.4**	5.1	5.8
18:2	35.4	27.0*	66.3	48.1**	55.8	49.8	15.0	8.1**	20.2	15.9*	2.4	1.5*
18:3	0.2	0.6**	0.2	1.5***	0.1	1.1***	0.5	1.1*	0.1	0.5**	-	-
20:0	0.1	0.1	0.2	-	0.2	-	0.1	-	-	-	21.5	20.5
20:1	0.1	4.0***	0.2	5.2***	0.3	5.6***	0.4	9.1***	0.1	3.4***	0.7	1.0
20:2	0.6	0.6	4.4	2.2*	4.8	2.4*	3.4	1.7*	0.9	1.2	-	-
20:4	5.7	4.1	0.4	0.7	3.3	0.7	13.3	7.2*	4.5	3.9	-	-
22:0	-	-	0.2	0.2	0.1	-	-	-	-	-	12.1	10.1
22:1	-	2.7	0.2	1.4***	0.2	1.8***	-	11.1	-	2.5	-	2.0
23:0	-	-	-	-	-	-	-	-	-	-	3.5	1.0*
24:0	-	-	-	-	-	-	-	-	-	-	10.7	11.8
24:1	-	-	-	-	-	-	-	-	-	-	4.5	9.0**

[a]TG = triglyceride; CL = cardiolipin; CER = cerebrosides; EPG = ethanolamine phosphoglyceride; CPG = choline phosphoglyceride; SPH = sphingomyelin.

[b]Number of carbon atoms: number of double bonds.

[c]Each value represents the mean of three animals per diet. Significant difference between diets at 5 (*), 1 (**) and 0.1% (***).

[d]DMA = dimethyl acetal.

TABLE III

Aliphatic Moieties in the 1 and 2 Position of Ethanolamine Phosphoglycerides (EPG) and Choline Phosphoglycerides (CPG) from Cardiac Lipids of Boars[a]

Chain length: number of double bonds	1-Acyl				2-Acyl			
	EPG		CPG		EPG		CPG	
	Corn	RSO	Corn	RSO	Corn	RSO	Corn	RSO
16:0	8.7	13.2	41.6	24.7	13.2	9.6	30.2	31.9
18:0	78.0	61.2*	47.2	47.2	42.2	33.6*	31.4	24.1
18:1	2.5	6.2*	1.2	5.6***	14.3	15.0	16.3	23.2
18:2	0.9	1.8	0.9	1.5	14.3	10.1	17.5	11.3
18:3	0.5	0.5	0.1	1.2*	0.1	0.1	-	0.2
20:1	0.1	2.1***	0.1	2.8***	0.8	12.4***	0.2	4.5***
20:2	0.2	0.1	0.1	0.3	2.0	1.9	0.7	0.8
20:4	0.4	0.5	0.4	0.1	7.3	5.3	1.7	1.2
22:1	-	4.4	-	5.4	-	8.7	-	2.7
22:4	-	-	-	-	1.0	0.3**	0.4	0.1
	1-Alkenyl ether				2-Acyl			
16:0	23.6	17.4*	55.1	38.3**	21.3	21.1	3.6	3.3
17:0	2.1	3.8	2.3	2.8	trace	trace	trace	trace
18:0	44.8	29.7**	24.0	22.2	20.1	13.4	1.7	2.7
18:1	20.2	27.1**	14.0	25.9**	9.3	9.9	16.1	23.0**
18:2	5.5	1.1**	4.2	1.1**	18.3	11.4	54.5	46.2*
18:3	-	-	-	-	-	0.3	-	2.1
20:0	1.4	1.6	0.4	0.6	trace	trace	trace	trace
20:1	0.2	2.4***	0.1	2.1***	0.7	9.3***	0.3	3.3***
20:2	0.7	2.2*	0.2	0.5	2.9	3.1	2.2	2.2
20:4	-	-	-	-	17.9	7.4**	17.1	11.3*
22:1	-	3.5	-	1.7	-	18.6	-	2.1
22:4	-	-	-	-	3.5	1.6*	1.9	0.6**

[a]Three boars per diet. Each value represents the mean of three animals. Significant differences between diets are indicated at the 5% (*), 1% (**), and 0.1% (***) level.

[b]Traces of fatty acids not included in both phosphatides: 14:0, 15:0, 17:0, 20:0, 20:3, 20:5, 22:5n-6, 22:5n-3, and 22:6. Traces of alkenyl ethers not included: 12:0, 14:0, 15:0, and 22:0.

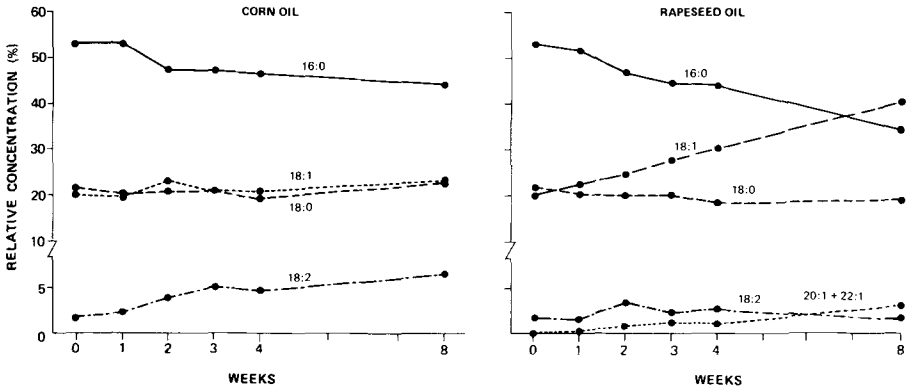

FIG. 1. The relative concentration of the major alkenyl ethers in the total cardiac lipids of boars fed diets containing corn oil or rapeseed oil for 8 wk.

chromatography with authentic standards, by hydrogenation and subsequent GLC analysis, and by prior separation by argentation-TLC (13) followed by GLC analysis.

The alkenyl ethers of the choline and ethanolamine phosphoglyceride were hydrolyzed by exposure to fumes of HCl (14) and the reaction products and unreacted phosphoglycerides were isolated by TLC using the solvent system $CHCl_3$:MeOH:H_2O 65:25:4. The relative concentration of alkenyl-acyls to diacyls was determined by the addition of methyl heptadecanoate as internal standard to the 2-acyl and diacyl phosphoglyceride. The diacyl phosphoglycerides were hydrolyzed enzymatically according to Weber et al. (15) to permit positional analysis. The relative abundance of alkyl-acyl to diacyl phosphoglycerides was obtained by reacting the unreacted phosphoglycerides above with $LiAlH_4$ and estimating the reaction products by TLC (16).

RESULTS AND DISCUSSION

As shown previously (6), heart weights and total cardiac lipids of boars fed diets containing either corn oil or RSO for 8 wk were not significantly different. Results from this study indicate that the cardiac phospholipids were not significantly affected (mg P/g of wet tissue: corn 0.36 ± 0.06, RSO 0.30 ± 0.03), but neutral lipids were significantly higher ($P<0.05$) when RSO was fed (% lipid: corn 30 ± 3, RSO 40 ± 6). A slight elevation of neutral lipids in hearts of pigs fed rapeseed oils has been observed previously (17,18).

The relative concentration of most polar lipids was remarkably similar in the two diets (Table I). Small differences ($P<0.05$) were observed between the relative abundance of choline and ethanolamine phosphoglycerides. In contrast, studies with rats indicate a decrease ($P<0.05$) of glycerophosphoryl ethanolamine (GPE) and an increase ($P<0.01$) of sphingomyelin in cardiac phospholipids (19).

The fatty acid composition of the major lipid classes is shown in Table II. When the diet containing rapeseed oil was fed, the relative concentrations of 18:1, 18:3, 20:1, and 22:1 increased in the total cardiac lipids (6). As seen in Table II, all subclasses showed the same trends, higher levels of these acids in pigs fed RSO compared to pigs fed corn oil. The highest concentration of long chain monoenes (20:1 and 22:1) was found in the ethanolamine phosphoglycerides. This is in marked contrast to the findings in rat heart lipids where a high concentration of 20:1 and 22:1 was found in triglycerides (2) and cardiolipin (7).

The relative abundance of the alkenyl-acyls to diacyls was not significantly affected by diet in either the ethanolamine (% of phospholipid: corn 40 ± 10%, RSO 42 ± 6%) or choline: (corn 45 ± 8%, RSO 39 ± 6%) phosphoglycerides. The amount of alkyl-acyls relative to diacyls in either phosphoglyceride was judged by TLC to be less than 5% from the presence of 1-alkyl glycerol after reduction of the unreacted phosphoglyceride obtained by acid hydrolysis with $LiAlH_4$. The contribution of the fatty acid of alkyl-acyl phosphoglycerides to the analysis of position 2 of the diacyl phosphoglyceride was therefore considered to be negligible.

The positional analysis of diacyl- and alkenyl acyl-choline phosphoryl ethanolamine (CPE) and -glycerophosphoryl choline (GPC) is shown in Table III. Characteristic differences were observed as expected between positions 1 and 2 and between the two phosphoglycerides. For example, the 1-position of the diacyl phospho-

glycerides was mainly saturated; 1-acyl-GPE contained predominantly 18:0, while 1-acyl-GPC contained equal amounts of 16:0 and 18:0. Position 2 of diacyl phosphoglycerides contained higher levels of unsaturated fatty acids compared to position 1. Differences were also apparent between position 2 of the alkenyl derivatives of both ethanolamine and choline phosphoglycerides which contained relatively more linoleate and arachidonate than the corresponding diacyl derivatives. The alkenyl group composition consisted mainly of 16:0, 18:0, and 18:1 moieties identified as aldehydes after acid hydrolyses. The 1-alkenyl-GPE was rich in the 18:0 moiety and 1-alkenyl-GPC contained high levels of the 16:0 derivative.

Feeding the diet containing rapeseed oil to pigs resulted in marked changes in the fatty acid composition of the ethanolamine and choline phosphoglycerides from pig heart (Table II). Significant amounts of long chain monoene fatty acids 20:1 and 22:1 were found in all acyl positions. Generally, the relative concentrations of 20:1 and 22:1 fatty acids were higher in position 2 compared to position 1 in both phosphatides; similar levels were found in position 1 between the two phosphatides; and higher levels were detected in 2-acyl-GPE than in 2-acyl-GPC. In an earlier study with liver phospholipids of rats fed the same rapeseed oil, 22:1 was also incorporated preferentially in position 2 of ethanolamine phosphoglyceride and choline phosphoglyceride, but 20:1 was found to be higher in position 1 of these phosphoglycerides (20).

The highest level of 22:1 was found in the 2-acyl position of alkenyl acyl-GPE. The accumulation of this acid could be due first to a slower enzymatic hydrolysis of erucyl esters compared to esters of common fatty acids (3). Indeed, this hypothesis has been used to explain the high levels of cholesteryl erucate in the adrenals of rats fed rapeseed oil (21). Secondly, acyltransferase activity of 1-alkenyl-phosphoglycerides was demonstrated to be low in many tissues examined (22,23) and may be further reduced by stress such as in the case of essential fatty acid deficiency (24). The lower enzymatic hydrolysis of erucate esters, and the possible stress on animals fed high fat diets (25), in particular, diets containing rapeseed oils high in 22:1 (26), could lead to an accumulation of this acid in alkenyl acyl-GPE.

As seen in Figure 1, the alkenyl group composition was influenced by the kind of fatty acids in the diet. Feeding a diet containing rapeseed oil resulted in a marked increase in the level of the 18:1 alkenyl ether moiety, and in the presence of 20:1 and 22:1 alkenyl ether groups. Analysis of the constituent alkenyl ethers of the two phosphoglycerides indicated a similarity in the level of 18:1, 20:1, and 22:1 alkenyl ethers. Although the composition of the major alkenyl constituents 16:0, 18:0, and 18:1 was strikingly different from that of the fatty acids on position 1 of the corresponding diacyl phosphoglycerides, a similarity in the level of 20:1 and 22:1 moiety was observed. These results clearly demonstrate that the erucate and eicosenoic esters can serve as precursers of alkenyl ethers.

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Lipid and Fatty Acyl Composition of Rat Brain Capillary Endothelia Isolated by a New Technique

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ABSTRACT

A method is described for the isolation of pure capillary endothelia from rat brain and the phospholipid composition of these cells is reported. This method is rapid and requires only a small amount of starting material. It involves: (a) tissue disruption by high speed homogenization, (b) separation of the capillary endothelia from other brain structures using sucrose gradients, and (c) a final purification using a glass bead column. Choline and ethanolamine phosphoglycerides were found to be the predominant lipid classes of these cells amounting to 31.9% and 24.4%, respectively, of total phospholipids. The choline phosphoglycerides consisted almost exclusively of 1,2-diacyl glycerophosphorylcholine, whereas the ethanolamine phosphoglycerides consisted of approximately equal amounts of 1,2-diacyl and 1-alk-1'-enyl-2-acyl glycerophosphorylethanolamine. The composition of the constituent fatty acids of both choline and ethanolamine phosphoglycerides and the alk-1-enyl composition of ethanolamine phosphoglycerides is reported. Saturated fatty acids accounted for 45% of the total fatty acids in choline phosphoglycerides and for 53% in ethanolamine phosphoglycerides. Arachidonic acid accounted for approximately 48% of the total fatty acids in alk-1-enyl ethanolamine phosphoglyceride.

INTRODUCTION

The transport of fluids and solutes from the blood to the central nervous system in mammals is controlled by a variety of mechanisms which collectively are known as the blood-brain-barrier (BBB). The capillary endothelia constitute an important component of this barrier system (1). A full understanding of their role in the BBB necessitates both a physiological and biochemical approach. The biochemical properties of these cells can best be studied on isolated fractions.

Several methods are available for the isolation of brain capillaries (2-6). However, the application of these procedures for routine use is limited by a requirement for large amounts of starting tissue (2,5,6), by a considerable length of time in their work-up (6), and by contamination from red blood cells (2,3) or other material (4). In this report, we describe a procedure for preparing highly purified capillaries from rat brain. The phospholipid composition and the fatty acyl composition of ethanolamine phosphoglycerides (EPG) and choline phosphoglycerides (CPG) from these fractions are presented.

MATERIALS AND METHODS

Isolation of Capillaries

Brain capillaries were prepared from Wistar (Woodlyn Lab., Ltd., Guelph, Ontario) rats of both sexes. Animals were decapitated, and the brains removed, weighed, and rinsed in ice cold 0.4 M sucrose containing 10,000 units of heparin per liter (Solution A). The cortex and cerebellum were trimmed of white matter, weighed, and rinsed again in Solution A (all operations carried out at 4 C). This tissue was chopped to a fine mince in a precooled petri dish containing a small volume of cold 0.4 M sucrose (Solution B). The mince was drained over 333 μm nylon mesh, scraped off and suspended in 60 ml of 0.4 M sucrose containing 20,000 units of heparin per liter and 0.1 mM ATP (Solution C). This suspension was homogenized (10 down/10 up) in a 30 ml loosely fitting (0.010 inch clearance) Potter-Elvehjem Teflon homogenizer using a Tri-R-Stir-R Model 563C variable speed laboratory motor (Tri-R Instruments, Rockville Centre, NY) at 12,000 rpm. The homogenized tissue was brought to 120 ml with Solution B.

The high speed used for homogenization should be noted. This is essential for the adequate disruption of the tissue. Care must be taken to maintain the clearance between the pestle and homogenizer wall to prevent grinding the tissue. If this precaution is not observed, the capillaries will be fragmented.

The capillaries were separated by centrifugation on sucrose gradients. The homogenized suspension was layered in 20 ml portions onto a discontinuous gradient prepared in 39 ml tubes

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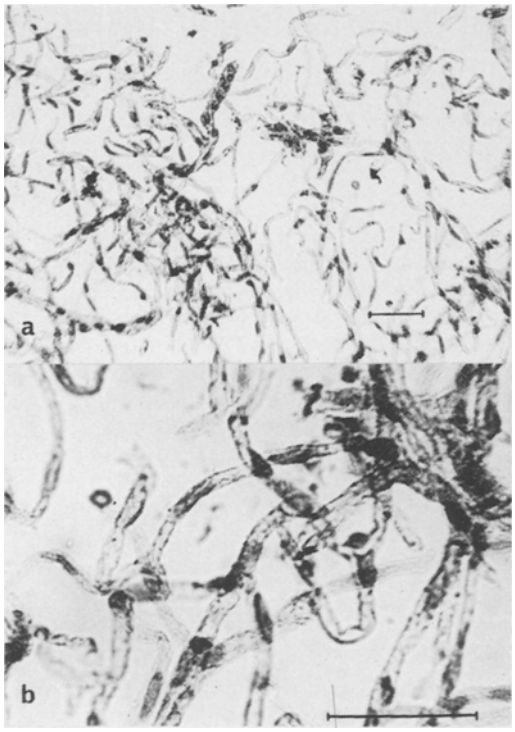

FIG. 1a. Photomicrograph of isolated rat brain capillary endothelia stained with methylene blue. Note freedom from contamination by nonvascular elements. FIG. 1b. Photomicrograph at higher magnification showing arborization and bifurcation of the capillary endothelia. Endothelial cell nuclei are prominent at this magnification. Note the absence of red blood cells in the vessel lumina. Scale lines = 50 μm.

for a Spinco SW-27 rotor. The gradient consisted of 6.9 ml of 1.0 M sucrose plus 1% bovine serum albumin (BSA) over 5.7 ml of 1.75 M sucrose plus 1% BSA. The tubes were centrifuged at 18,500 rpm (41,000 g) for 30 min. All of the material at the 1.0 M/1.75 M interface and the 1.75 M sucrose layer, including the pellet, were collected and brought to 60 ml with Solution B. This suspension should be checked by light microscopy for the presence of capillaries. If homogenization has been faulty, they will have been destroyed and there is no point in proceeding further. This solution was homogenized gently (5 down/5 up) at 500 rpm again taking care to maintain the clearance between the pestle and the wall of the homogenizer. The suspension was brought to 120 ml with Solution B and layered in 20 ml portions onto a discontinuous gradient consisting of 6.9 ml of 1.2 M sucrose plus 1% BSA, over 5.7 ml of 1.65 M sucrose plus 1% BSA. The tubes were centrifuged at 18,500 rpm (41,000 g) for 30 min. The material at the 1.2 M/1.65 M interface and the 1.65 M layer, including the pellet, were collected. This mixture was diluted to 100 ml with a solution of 0.4 M sucrose containing 0.1 mM $CaCl_2$, 0.1 mM ATP, and 0.4 mM 2 N-morpholinoethanesulfonic acid buffer (MES) pH 6.1 (Solution D). This suspension containing capillaries, free nuclei, and fine cellular debris was passed through a 333 μm nylon screen. The screen was rinsed forcefully with Solution D. The resulting suspension should be checked for capillaries as before.

The crude capillary suspension was purified by passing it through a 100 ml, 45-60 mesh glass bead (Potters Industries, Inc., Carlstadt, NJ) column (Plexiglass 55 mm dia x 120 mm ht made in the Erindale College workshop) covered at one end with 153 μm nylon mesh. The outlet of the column was ca. 1 cm in diameter. A piece of tubing was attached to the outlet and partially closed with a screw clamp so that the flow through the column was slow. The capillaries were retained on the column while the nuclei and small cellular debris were washed through the column with three 100 ml portions of Solution D. The contents of the column were poured into a beaker and suspended in 100 ml of Solution D. Capillaries were separated from the beads by agitation with a glass rod. Once the beads had settled, the suspension containing the capillaries was poured off. This step was repeated two or three more times or until all the capillaries were removed from the beads. The capillaries were further purified by passing the suspension through a second glass bead column, prepared in the same manner and the entire purification proeedure repeated. The final suspension of purified capillaries was concentrated by centrifugation at 7000 g for 30 min.

Heparin was added to Solutions A & C to prevent blood clotting and to facilitate and removal of blood. The addition of ATP (Solutions A, C & D) helps to maintain the metabolic integrity of the endothelial cells.

MES was chosen as the buffer since it has little effect on cellular integrity and does not bind metal ions. Moreover, it does not interfere with cellular metabolites as for example does orthophosphate (phosphate buffers).

Electron Microscopy

Samples of pelleted capillaries were fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.2 for 1 hr, rinsed in buffer, postfixed in 1% osmium tetroxide in phosphate buffer for 1 hr, and dehydrated in a graded series of ethanol (15 min in each). The material was then stained in 1% uranyl acetate in 100% ethanol for 1½ hr (7) before infiltration and embedding in Spurr's

low viscosity medium (8). Silver-gold sections were cut with an LKB III ultramicrotome, mounted on uncoated 200 mesh copper grids, stained with lead citrate (9) and examined in an Hitachi HU-12 electron microscope at an accelerating voltage of 75 kilovolts.

Lipid Extraction and Analysis

Total lipids were extracted from purified capillaries (10) and the phospholipids separated by two-dimensional thin layer chromatography (TLC) as previously described (11). Plasmalogens were determined by the procedure of Horrocks (12). Lipid phosphorus was determined using the method of Eng and Noble (13).

Analysis of Fatty Acids and Dimethyl Acetals

The individual phospholipids were separated by separation-reaction-separation TLC (12) or by TLC without acid hydrolysis (11) when dimethyl acetals were to be analyzed. After development in the second dimension, the lipids were visualized by spraying with 2',7'-dichlorofluorscein and viewing under ultraviolet light. The EPG and CPG spots were scraped from the plate and subjected to methanolysis by heating in 4% H_2SO_4 in methanol in sealed vials at 90 C for 90 min. The fractions were purified and the dimethyl acetals separated from the methyl esters on Silica Gel G plates developed in benzene.

The fatty acid patterns of EPG and CPG were determined with a Hewlett-Packard 7026A research gas chromatograph. The samples were injected onto a 6 ft glass column (3 mm ID) packed with 15% Hi Eff 2BP on 80/100 mesh Gas Chrome P (Applied Science Laboratories, Inc., State College, PA). The operating column temperature for methyl ester analysis was kept in isothermal at 180 C. Dimethyl acetals were analyzed at 150 C. Peaks were identified by comparison of relative retention times with those of standards obtained from Supelco, Inc., Bellefonte, PA, and by determination of equivalent chain length according to Hofstetter et al. (14). Peak areas were determined with a Hewlett-Packard 3380A integrator. Quantitative results with fatty acid standard F (NIH Mixture) differed from the stated composition by a relative error of less than 6% for the major components (>10% of mixture) and less than 5% for minor components (<10% mixture). These values for the percent relative error are the means of six determinations.

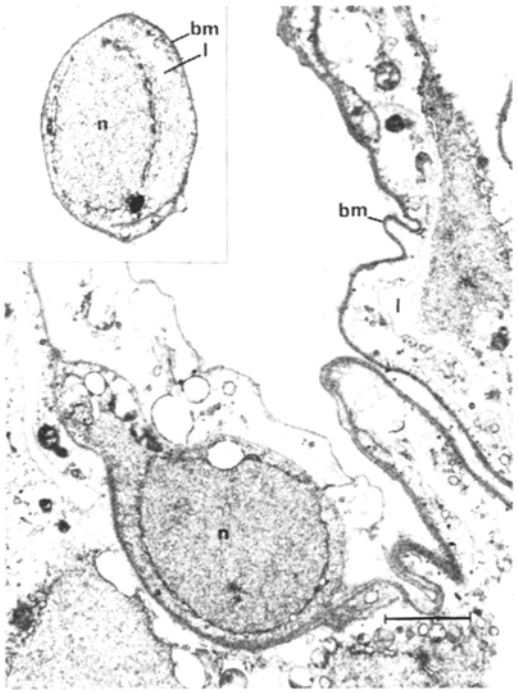

FIG. 2. Electron micrographs of capillary preparation showing typical ultrastructural features of capillaries. Capillaries are shown in longitudinal section and transverse section (inset). n = nucleus, bm = basement membrane, l = lumen. Note the absence of red blood cells in the lumina. Scale line = 2 μm.

RESULTS AND DISCUSSION

Isolated capillaries were examined by light

TABLE I

Phospholipid Composition of Rat Brain Endothelial Cells[a]

Lipid class	% of total lipid phosphorus
Ethanolamine phosphoglycerides[b]	24.4 ± 1.9
Choline phosphoglycerides	31.9 ± 3.4
Serine phosphoglycerides	9.8 ± 0.9
Sphingomyelin + inositol phosphoglycerides	18.1 ± 0.8
Phosphatidic acid	0.9 ± 0.1

[a]Results are the means from three separate isolations, mean ± standard deviation.
[b]46.3% plasmalogen.

TABLE II

The Composition of Constituent Fatty Acids (wt %) of Ethanolamine Phosphoglycerides and Choline Phosphoglycerides from Rat Brain Endothelial Cells[a]

	Ethanolamine phosphoglycerides		Choline phosphoglycerides
Acyl group	Alk-1-enyl acyl[b]	Diacyl[c]	Diacyl[c]
16:0	3.5 ± 0.8	14.0 ± 0.1	35.0 ± 0.9
16:1	tr	1.5 ± 0.2	0.6 ± 0.1
18:0	1.9 ± 0.1	31.0 ± 0.4	17.9 ± 1.7
18:1	20.0 ± 0.2	17.7 ± 0.3	23.4 ± 2.6
18:2n-6	2.0 ± 0.1	6.5 ± 0.1	8.6 ± 1.7
20:0	0.8 ± 0.3	0.6 ± 0.1	0.3 ± 0.1
20:1	5.3 ± 0.6	1.2 ± 0.1	1.3 ± 0.2
20:2	0.7 ± 0.3	tr	0.2 ± 0.1
20:3n-6 20:3n-9	0.9 ± 0.4	0.9 ± 0.1	0.8 ± 0.2
20:4n-6	48.3 ± 1.6	17.1 ± 1.0	10.2 ± 0.4
20:5n-6	1.3 ± 0.1	tr	tr
22:4n-6	5.7 ± 0.2	2.1 ± 0.0	tr
22:5n-6 22:5n-3	1.4 ± 0.3	1.1 ± 0.1	tr
22:6n-3	9.6 ± 0.4	6.7 ± 0.3	1.4 ± 0.4

[a]All analyses were made in duplicate on three separate isolations, mean ± standard deviation.

[b]Duplicate determinations on two separate isolations.

[c]Includes small amount of alkyl acyl.

TABLE III

Alkenyl Group Composition of Ethanolamine Phosphoglyceride from Rat Brain Endothelial Cells

Alkenyl group[a]	Weight %
16:0	21.6 ± 0.2
16:1	1.2 ± 0.4
17:0	1.6 ± 0.1
18:0	46.4 ± 0.5
18:1	26.9 ± 0.9
18:2[b]	1.8 ± 0.2
Others	0.5 ± 0.2

[a]Analysis in duplicate of two separate cell isolations, mean ± standard deviation.

[b]Tentative identification on the basis of equivalent chain length.

microscopy at low (Fig. 1a) and high (Fig. 1b) magnification and found to be free from contamination by other brain structures. Moreover, the vessel lumina were free of contamination from red blood cells. The isolated fragments were characterized by considerable aborization, by bifurcation of the vessels, and by prominent endothelial nuclei (Fig. 1b). Examination by electron microscopy (Fig. 2) confirmed the absence of erythrocytes and showed that the structure of the capillaries was well preserved.

The average yield of lipid was 3.56 ± 0.53 mg based on 12 g (wet wt) of starting material. Values for individual phospholipids shown in Table I were similar to those reported previously for human and bovine brain endothelia (6). Two dimensional TLC of the capillary lipids showed very small amounts of material with the chromatographic behavior of galactolipids. These results suggest little or no contamination by myelin. Myelin was eliminated by the use of sucrose gradients. Due to its high lipid-protein ratio, myelin will not sediment in sucrose gradients with a molarity greater than 0.8 M. The absence of myelin was also verified by electron microscopy (Fig. 2).

The constituent fatty acids of diacyl EPG and CPG are shown in Table II. The saturated fatty acids, 16:0 and 18:0, were found to be the major fatty acids of diacyl EPG and CPG, amounting to 45% and 53%, respectively. The greatest difference between the fatty acid compositions of these phospholipids was the reversal of the 16:0 to 18:0 ratio and the higher concentration of polyenoates in diacyl EPG. Alk-1-enyl EPG, in contrast, contained a greater amount of 20:4 n-6 fatty acids (Table II).

The alkenyl group composition of EPG (wt %) was found to resemble that of mouse microsomes, mitochondria, and human erythrocytes (Table III). The high content of 20:4 is similar to that of human gray matter (15). Comparable results for both the fatty acyl and alkenyl group composition of brain capillary endothelia have not been reported previously.

This report describes a three step procedure for the isolation of rat brain capillary endothelia: (a) tissue disruption using high speed homogenization with a loosely fitting Teflon homogenizer, (b) removal of myelin and less dense particulate matter in two, two-step sucrose gradients, and (c) the use of glass beads for the final purification of the capillaries. The method is rapid, requiring ca. 3 hr to complete. In addition, the procedure can be performed with a small amount of starting material (7 brains/isolation) and can be used with immature or adult rats. From the examination of light and electron micrographs, we conclude that the capillary endothelia prepared by this procedure are quite pure. In particular, the vessel lumina were free of red blood cells, yielding cells suitable for studies of membrane composition and the role of lipids in transport mechanisms.

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Potential for Employing the Distribution of Anomalous Non-Methylene-Interrupted Dienoic Fatty Acids in Several Marine Invertebrates as Part of Food Web Studies

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ABSTRACT

A group of homologous, nonmethylene-interrupted dienoic fatty acids (NMID) recently reported in oysters has been found in several other shellfish species and also in other marine phyla. The distribution of most other "normal" fatty acids among several species of shellfish is basically similar but mantle lipids from two other molluscan species, both squid, differ radically. The squid mantle fatty acids do not include NMID, suggesting that in molluscs NMID may accumulate primarily in filter-feeders or herbivores. The distribution of these anomalous fatty acid components in higher species suggests that they reflect invertebrates in the diet and are biochemically inert.

INTRODUCTION

Comparative studies of fatty acids in marine molluscs include studies from this laboratory (1-4), Italy (5,6), Japanese papers (e.g. 7-9 plus others too numerous to list) and a variety of other data, some of which is tabulated by Voogt (10). The relations between fatty acid compositions and diet or environment have often been complicated by occasional reports of high proportions of eicosadienoic and docosadienoic fatty acids imputed to be 20:2ω6 and 22:2ω6[3], although these particular acids are seldom significant in marine animal depot fats or fish muscle lipids (11). We have recently shown that certain unusual dienoic components are homologous series of C_{20} and C_{22} nonmethylene-interrupted (NMID) fatty acids (12). Thus, the major isomers in the American oyster *Crassostrea virginica* were found to be: 20:2Δ5,Δ13 and 20:2Δ5,Δ11; 22:2Δ7,Δ15 and 22:2Δ7,Δ13.

This type of unsaturation rarely occurs in nature. Similar fatty acids have previously been observed in a few seaweeds (13) and terrestrial plants (14-16) as enumerated in a review by Pohl and Wagner (17). In higher animals, NMID fatty acids generally occur as minor components in specialized lipids (18), or as the result of certain dietary restrictions (19,20). Their occurrence in molluscs (1,4) was thought to possibly represent a phylogenetic characteristic of molluscs since an arthropod from the first environment studied showed little or no NMID (1). Later we found NMID in marine sturgeon fats (21) and have therefore examined a number of phyla to determine if this occurrence could be specifically linked to molluscs.

Our survey has revealed the presence of NMID fatty acids, with the same chemical characteristics as those found in the North American oyster *C. virginica,* in several additional molluscs, an arthropod, a coelenterate, an echinoderm and in one or more species of marine vertebrate, but not in mantles of two species of squid. The findings tend to support an explanation that the distribution of NMID fatty acids is based on relationships within the food web and that the fundamental origin or accumulation may be in any of several invertebrate phyla. It is suggested that these acids could be much more useful than common fatty acids in examining food web relationships.

EXPERIMENTAL PROCEDURES

All animals collected by the authors for this study were obtained from coastal or offshore waters of the maritime provinces of Canada as follows: North American oysters *C. virginica* and European oysters *Ostrea edulis* from Ellerslie, Prince Edward Island; mussels *Mytilus edulis* and the plant *Ascophyllum nodosum* from Jeddore Harbour Nova Scotia; scallops *Placopecten magellanicus,* squid *Loligo pelaii,* and starfish *Asterias vulgaris* from coastal waters of Nova Scotia; and quahaugs *Venus mercenaria* from Passamaquoddy Bay, New

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[3]Shorthand notation for chain length: number of ethylenic double bonds and position of ultimate double bond relative to terminal methyl group. This notation also implies one methylene group between ethylenic double bonds. In the Δ system each bond is designated relative to the carboxyl carbon.

Brunswick. Other samples of lipid were obtained from concurrent studies and are described in other publications. Extraction of lipids where necessary was carried out with chloroform and methanol by the method of Bligh and Dyer (22). Lipids were saponified and unsaponifiable materials were removed by procedure Ca-6b-53 of the AOCS. Recovered fatty acids were converted to methyl esters by refluxing with methanol:boron-trifluoride reagent (7%). Where required, fatty acid methyl esters were subjected to thin layer chromatography (TLC) on silica gel impregnated with 25% $AgNO_3$, using reagent grade chloroform, redistilled in a simple glass still, as the developing solvent. Fractions eluted from the silica with the same chloroform were examined by gas liquid chromatography (GLC) using stainless steel open tubular columns, 45 m in length x 0.25 mm ID, coated with butanediolsuccinate polyester and operated in a Perkin-Elmer model 900 machine. Operating conditions for GLC are described elsewhere (1). The tentative identification of NMID fatty acids in the species surveyed is based primarily on coincidence of obvious "unknown" components in total fatty acid methyl ester analyses with components identified in detail as NMID in oysters (Fig. 1), supplemented by $AgNO_3$/TLC where required by low levels of NMID in some species.

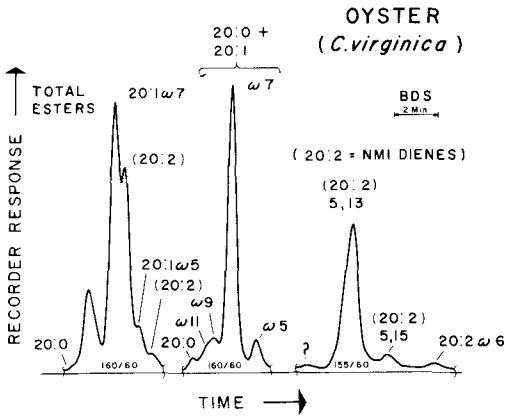

FIG. 1. Parts of open-tubular gas chromatography charts from analyses of methyl esters of oyster total lipid fatty acids. From left to right: total 20:0–20:1 region; band for monoethylenic acids from $AgNO_3$-TLC; dienoic band. Analyses on BDS-coated column basically as described by Ackman and Hooper (1). Temperature and pressure 160 C and 155 C, 60 psig, as indicated.

RESULTS AND DISCUSSION

Fatty acid analyses for several of the species referred to in Table I have been reported on elswhere in other contexts and greater detail: *C. virginica* and *O. edulis* by Watanabe and Ackman (4); *Ascophyllum nodosum* by Jamieson and Reid (13); the Mexican Ridley turtle, *Lepidochelys kempi* by Ackman et al. (23); the common periwinkle *Littorina littorea,* the moon snail *Lunatia triseriata,* and the sand shrimp *Crangon septemspinosus* by Ackman and Hooper (1); the squid, *Ilex illecebrosus* by Jangarrd and Ackman (24), the sturgeon *Acipenser oxyrhynchus* by Ackman et al. (21), and the white jellyfish *Aurelia aurita* by Hooper and Ackman (25).

Gross similarities among the fatty acid compositions of the seven species of shellfish given in Table I are quite evident. The total amounts of saturated acids range from 28.5 to 36.7%, of monounsaturated acids from 15.2 to 27.9%, and of polyunsaturated acids from 41.7 to 53.9%. Among the percentages of individual components, the low percentages of 22:6ω3, of high 18:3ω3 and 20:3ω3, or of high 18:2ω6 and 20:4ω6, in the herbivorous gastropod *L. littorea* deserve mention. The other shellfish are exposed to varied diets, probably mostly of different unicellular algae, despite which it has been proposed that small differences in composition are based on specific species needs (4). These results for the highly unsaturated acids are in general agreement with the fatty acid compositions in the survey of Adriatic species by Cerma et al (6). However, the differences between these surveys are quantitative rather than qualitative and are possibly a result of seasonal variations (3,5). The normally cold waters of the Nova Scotia coast do not seem to impose a requirement for higher levels of polyunsaturated fatty acids. It is of interest that unknown components listed by these authors for many species of molluscs could be NMID.

The two analyses of squid mantle fatty acids pose the question of whether phylogenetic biochemical similarities exist in the fatty acids of the Mollusca as a phylum. The two species of squid have very similar fatty acid compositions, which differ significantly in certain details from those of the shellfish. Levels of 16:0 in the squid are 50% to 100% greater than in the shellfish while levels of 16:1 are much lower. Percentages of 22:6ω3 are at least twice as high in squids as in other molluscs, while the acids of basic plant origin, 18:2ω6 and 18:3ω3, and 20:4ω6 the successor of 18:2ω6, are much lower. Nonlipid components such as glycogen have also been compared for squid and other molluscs (26).

The assumption of different trophic food web relationships between squid and the shellfish, which feed almost exclusively by filtration

TABLE I

Basic Compositions, in Some Marine Animals and a Plant, of Total Methyl Esters of Saturated, Monounsaturated, and Polyunsaturated Fatty Acids[a]

Phylum, species and reference	Fatty Acids															
	% Saturated										% Monounsaturated					
Mollusca	14:0	15:0	16:0	17:0	18:0	20:0	Other linear	4,8,12-TMTD	Other branched chain	Total	16:1	18:1	20:1	22:1	Other	Total
C. virginica	2.1	1.0	19.1	1.5	4.3	0.1	0.2	0.8	1.8	30.9	2.4	5.4	6.6	0.2	0.6	15.2
O. edulis	3.0	0.9	20.8	1.0	4.0	–	0.2	0.2	3.0	33.1	3.3	7.3	4.1	1.0	1.1	16.8
M. edulis	3.2	1.0	15.9	1.0	3.7	0.2	0.6	2.3	2.5	30.4	7.5	11.1	7.5	1.4	0.4	27.9
P. magellanicus	3.1	0.9	16.6	1.1	4.5	–	0.6	2.3	1.8	30.9	5.7	9.5	3.3	–	0.9	19.4
V. mercenaria	2.0	0.4	12.3	1.1	5.5	0.4	0.8	2.2	3.8	28.5	4.8	8.4	8.6	1.5	1.0	24.3
L. littorea	6.0	0.4	13.6	0.8	4.8	0.1	0.3	4.0	1.1	31.1	3.5	4.5	6.6	0.4	2.1	17.1
L. triseriata (1)	4.9	0.8	13.0	1.2	10.4	0.8	2.9	1.4	1.3	36.7	3.4	7.5	7.2	0.5	0.7	19.3
L. pelaii, mantle	2.0	0.7	28.2	0.6	4.5	–	–	0.1	1.2	37.3	0.5	4.1	4.2	0.3	0.2	9.3
I. illecebrosus, mantle (24)	2.2	0.3	27.6	0.3	4.4	–	–	–	–	34.8	0.4	4.9	4.9	0.5	0.1	10.8
Echinodermata																
A. vulgaris	3.9	0.8	6.9	0.6	3.3	0.1	0.9	0.6	3.6	20.7	2.7	7.7	19.3	1.1	0.6	31.4
Vertebrata (depot fat)																
L. kempi (23)	12.4	0.6	18.4	0.7	5.6	0.3	1.1	0.1	1.8	41.0	14.1	26.3	2.1	0.2	1.0	43.7
A. oxyrhynchus (21)	3.8	0.7	15.2	0.3	2.0	0.2	0.2	0.1	3.6	26.1	6.5	30.1	6.7	1.0	1.3	45.6
Arthropoda																
C. septemspinosus (1)	1.4	1.1	20.1	1.6	7.6	0.3	0.3	0.2	1.8	34.3	6.0	16.2	1.3	0.3	2.0	25.8
Coelenterata																
A. aurita (25)	3.3	1.9	16.0	0.7	6.4	1.2	0.7	0.1	3.0	32.4	4.7	11.8	13.7	4.5	3.3	38.0
Phaeophyta																
A. nodosum	7.4	–	9.8	–	0.8	–	0.2	–	–	18.2	1.8	30.4	0.2	0.8	0.2	33.4

Phylum, species and reference	% Polyunsaturated														% NMID	
Mollusca	16 poly	18:2ω6	Σ20:2	Σ22:2	18:3ω3	20:3ω3	18:4ω3	20:4ω6	22:4ω6	20:5ω3	22:5ω3	22:6ω3	Other	Total	20:2	22:2
C. virginica	0.8	1.7	1.9	5.8	2.8	0.3	2.6	4.2	0.4	12.0	2.0	18.6	0.8	53.9	1.71	5.79
O. edulis	0.2	2.5	0.5	1.7	4.6	0.2	5.0	3.2	1.4	19.9	0.4	9.8	0.7	50.1	0.22	1.55
M. edulis	0.8	1.0	4.3	2.9	1.1	0.2	1.9	3.8	0.3	10.2	1.2	13.7	0.3	41.7	4.04	2.86
P. magellanicus	1.4	0.9	0.7	0.1	0.9	0.5	4.2	2.9	–	19.3	0.2	18.4	0.2	49.7	0.21	0.05
V. mercenaria	0.9	0.2	3.8	0.3	0.9	0.1	1.1	3.6	0.4	18.3	2.0	15.0	0.6	47.2	3.61	0.25
L. littorea	2.6	2.6	2.2	1.7	6.5	2.5	3.5	7.9	–	18.1	2.3	0.2	1.7	51.8	0.35	1.67
L. triseriata (1)	–	1.0	2.4	4.4	0.2	0.2	0.2	5.1	1.0	13.4	3.2	7.7	5.2	44.0	0.60	4.01
L. pelaii, mantle	0.1	0.2	0.1	–	0.1	0.5	0.1	0.8	0.1	14.3	0.3	36.4	0.4	53.4	<0.01	<0.01
I. illecebrosus, mantle (24)	0.3	0.3	0.3	–	0.1	–	0.1	0.8	0.1	15.8	0.3	37.1	–	55.2	<0.01	<0.01
Echinodermata																
A. vulgaris	0.8	0.4	6.6	1.6	0.6	0.3	0.8	4.7	0.1	18.8	1.1	10.9	1.2	47.9	5.67	1.56
Vertebrata (depot fat)																
L. kempi (23)	0.1	0.5	0.9	0.8	0.4	0.5	–	2.4	0.9	1.6	2.2	4.1	1.1	14.5	0.62	0.80
A. oxyrhynchus (21)	2.3	0.9	0.8	0.2	1.4	0.8	1.6	2.7	0.6	10.2	2.9	3.3	0.5	28.2	0.48	0.52
Arthropoda																
C. septemspinosus (1)	–	2.4	0.2	2.1	0.6	0.2	0.3	4.2	0.2	18.7	0.9	11.7	0.8	40.3	0.06	0.13
Coelenterata																
A. aurita (25)	–	0.8	2.3	0.1	0.4	–	0.1	6.7	0.6	8.5	1.6	7.0	0.7	28.8	1.3	0.1
Phaeophyta																
A. nodosum	0.6	7.7	2.0	–	5.3	4.5	4.4	14.0	0.2	8.8	0.6	0.1	–	48.2	(20:3 NMI; 0.1-0.4)[b]	

[a]The dienoic polyunsaturated fatty acids are shown as the total of methylene-interrupted types and NMID types, and the latter are then shown separately (in weight percent)

[b]4 Other species, 7 analyses (13)

of algae from sea-water, would seem to be a good alternative explanation for the differences between these two species and the rest of the Mollusca examined. The squid are exclusively carnivorous, and thus resemble many fish in behavior (27), in being mobile, and in preying on small fish and crustacea. As the latter presumably do not include NMID in their fatty acids, the NMID do not accumulate in the squid fatty acids. A similar reasoning explains why cholesterol is at least 90% of the total sterol in *I. illecebrosus* (28), but is often much less in proportion of sterols in the filter-feeding molluscs (10,29-32). It should be noted that the moon snail, *L. triseriata,* is an active and obligate carnivore rather than a filter feeder. It is nonetheless possible that *L. triseriata* has a close trophic link to algae since its major prey in the location of collection is the filter-feeding clam *Mya arenaria* (1).

Included in Table I is an analysis of the alga, *A. nodosum,* closely associated with the particular lot of mussels *M. edulis.* This alga may not itself be a good source of fatty acids for filter-feeding shellfish except in its spore stage of development or later as organic detritus (33). It may, however, be taken as representing a widely distributed food material available to marine invertebrates in Nova Scotia littoral waters. Also included in Table I are an obvious shellfish predator, the common starfish *A. vulgaris,* and depot fats from a turtle, *L. kempi,* and a fish, *A. oxyrhynchus,* both of which include shellfish in their diets. The basic fatty acid compositions of these three diverse types of animals fail to indicate any trophic relationship. In fact, the fatty acid distirbutions of these three species and of the shellfish are notably different in almost all quantitative aspects. Although qualitatively they share the usual marine oil fatty acids, only the NMID indicates a common and unique link between prey and predator (Table I).

These findings are not overly surprising. As Watanabe and Ackman (4) have demonstrated in feeding experiments with oysters, there appears to be a "species-oriented" fatty acid composition superimposed on the typical marine fatty acid pattern, which cannot easily be altered by dietary factors in the absence of any large amount of depot fat. These authors suggested that the oysters rapidly converted algal fatty acids in such a way as to maintain this species-oriented composition. Such a semirigid requirement for fatty acid composition may also hold true for the starfish, but is less likely in the turtle and sturgeon where massive depot fat accumulation takes place. Squid do possess this type of fat in the liver (24,34) further

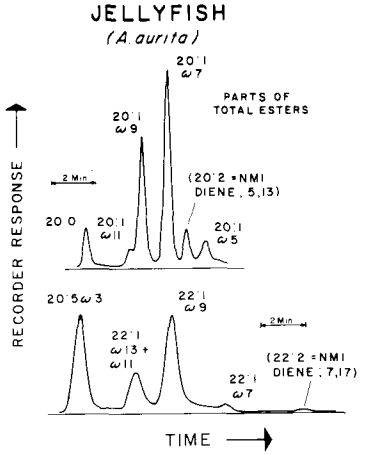

FIG. 2. Parts of open-tubular gas chromatography charts from an analysis of methyl esters of jellyfish total (*A. aurita*) lipid fatty acids. Above: 20:0-20:1 region; below 20:5ω3-22:1 region. Column and conditions similar to Figure 2, Ackman and Hooper (1).

setting them apart from other molluscs. However, even in cephalopods, dietary preferences may be a major factor since NMID were not reported in fatty acids of several squid (35) whereas a 22:1(?) has been listed in an octopus which presumably could be preying on molluscs (36).

If a fatty acid component is not subject to rapid metabolic turnover, then it will presumably accumulate as a result of continued dietary intake and may well be passed along the food chain. Our survey of NMID fatty acids in Table I illustrates the distribution in the species discussed above. The various components were qualitatively common to all the shellfish as well as their predators. They are, however, absent from the carnivorous squid and are present in only very small proportions of the fatty acids of the arthropod *Crangon septemspinosus,* which feeds on animal tissue or detritus (37). The jellyfish *Aurelia aurita* also contains NMID fatty acids (Fig. 2). It presumably shares with the squid a semicarnivorous diet of small fish and pelagic invertebrates. However, it would be premature to discount coelenterates as primary producers of NMID. The absence of sufficiently detailed technology in analyses published for other species makes a thorough comparative survey of the literature difficult to achieve. A recent analysis of the bivalve *Yoldia limatula* by Farrington et al (38) suggests that these animals may also contain NMID fatty acids, as the high levels of 22:2 is otherwise difficult to explain. It also seems quite likely that NMID components are present in at least eleven additional

species of molluscs investigated in Italy (6). A detailed study of fatty acids in the gonad of the sea-urchin *Anthocidaris crassispina* showed 9.9% unidentified 22:2 isomers in the fatty acids of the nonpolar lipid, as well as a 22:2 at 3.5%, and it is a reasonable assumption that these are NMID (39). Other marine invertebrate studies support this work of Kochi in respect to sea urchins (40-42), a urochordate (43), and a holothurian (44) although in proportions of 20:1, 20:2 or 22:2 and not specifically in terms of NMID. Noguchi et al. (45) examined five gastropods and report 0.8-6.6% 22:2, while 10 bivalves had 1.2-13.5%. The sponge *Microciona prolifera* studied by Jefferts et al. (46) contains a different type of NMID of C_{26} chain length.

It may be significant that the structure indicated for the NMI trienoic fatty acid in algae by Jamieson and Reid (13) is 20:3Δ5, Δ11, Δ14. This material could presumably be produced by basic biochemical routes requiring that a Δ^5 desaturase act on a Δ^{11} monoene C_{20} acid (20), thus accounting for the 20:2Δ5, Δ11 found in oysters in our earlier study (12), but this is distinct from the desaturation documented for bacilli by Quint and Fulco (47) for the Δ^5 (and Δ^{10}) positions and reviewed in detail by Witting (48). There is some evidence for a chain elongation process occurring since in oysters the specifically identified 20:2 NMID acids were converted to 22:2 NMID acids (12), but we did not find (1) any special indication of any Δ^8 desaturation of C_{20} NMID to the corresponding 20:3. In the rat, in circumstances of deprivation of essential fatty acids, a 20:3ω9 is formed, but it is methylene-interrupted as discussed in some detail by Witting (48) or by Ullman and Sprecher (20) and Egwim and Sgoutas (19) for circumstances which do not apply here. The findings of Jefferts et al. (46) strongly suggest that, under some circumstances, desaturation of NMID components in marine life-forms may continue to multiple unsaturation but not in a normal methylene-interrupted manner. The likelihood of continued metabolism, anabolic or catabolic, has not been widely discussed. If such processes, as a result of the unusual structure of these components, occur relatively slowly, then NMID fatty acids may well accumulate in fatty predators with greater ease than is the case with some common fatty acids. The occurrence of NMID in body (depot) fats of *L. kempi*, and in *A. oxyrhynchus* depot fat, is very reasonable evidence for accumulation due to inertness. Had NMID originated in other than molluscs it seems likely that they would have been discovered earlier except for the fact that few fish in which the depot fat has been examined prey extensively on molluscs.

The presence of plentiful "normal" polyunsaturated fatty acids in shellfish (Table I) suggests at least some degree of limitation of formation of NMID components by competition even if NMID are substitute "essential" polyunsaturated fatty acids as suggested earlier (1). Unpublished work from this laboratory indicates that NMID in oysters are higher as a percentage of fatty acids under either non-feeding conditions or when the oysters were fed a diet lacking polyunsaturated fatty acids (D. Trider, personal communication). However, it is not clear if this is due to active biosynthesis or resistance to catabolism.

It is also possible to speculate that the NMID occur in outer cell membranes of molluscs in areas exposed to microbial attack. The unusual positions of the ethylenic unsaturation could hinder hydrolysis of lipids by lipases. In the 18:1 acids, the ω7 and ω5 isomers are susceptible to pancreatic lipase (49,50). On the other hand, proximity of a double bond to the carboxyl group confers this resistance (49,51). Other properties may be involved (52-54). Fujino et al. (40) observed that their 22:2 fatty acid remained bound during natural curing of sea urchin gonads in salt whereas other fatty acids were liberated during this process. This is possibly a factor in the above hypothesis of resisting microbial attack or lipases. A resistant membrane structure could be involved in phagocytosis which is an important aspect of food assimilation by filter-feeders (55).

The usefulness of the unusual NMID fatty acids in tracing the diversities of the food web are quite evident. Relationships which are beyond detection by analysis of normal fatty acids become obvious from the observation of NMID fatty acids in Table I. NMID fatty acids may become an effective tool in determining the maximum number of steps to which lipids of primitive organisms may influence a food chain. Not all food web problems are resolved by this limited survey. For example, if Kochi (39) found actual NMID in a herbivorous enchinoderm, it would have to be shown if it was basically of the type of NMID which we postulate for *A. vulgaris* to be either from de novo biosynthesis or from shellfish prey, or of the type shown to originate in algae by Jamieson and Reid (13).

Both the Mexican Ridley turtle *L. kempi* (23) and the Atlantic sturgeon *A. oxyrhynchus* (21) are loosely described as feeding on bottom invertebrates. We also showed through fatty acids of algal origin that the sturgeon probably included algae in their diet. Although the proportions of NMID (Table I) do not enable

one to distinguish among molluscs, echinoderms, or algae as potential sources for the sturgeon NMID, a selective examination of proportions of NMID component might be more rewarding. To add to the complexity of the problem in this case, the marine sturgeon is probably active in laying down depot fat through the biosynthesis of 16:0, leading to 16:1ω7 and to high proportions of 18:1ω7 in liver polar lipid and of 20:1ω7 in depot fat. It is not impossible that the 20:2 NMID are chain extended to some degree by higher animals. Thus, 22:2 is as important as 20:2 in the sturgeon depot fat and both the depot fat and the liver polar lipids showed 22:1ω7 to be higher than usual in marine oils as a proportion of 22:1, although total 22:1 was minor ($\leqslant$1%). Care must be taken in sampling for the type of analysis employed in this type of food chain work. Our initial analysis (1) of whole sand shrimp *C. septemspinosus* total lipid showed no obvious NMID, although a later, more refined, analysis showed low levels in the whole animal (Table I). From this meager study on one species, we would not associate NMID with shrimp or marine crustacea in general, but a recent analysis of shrimp head (*Penaeus aztecus* and *Penaeus setiferus*) has shown that NMID are quite plentiful compared to 20:1 and 22:1 (J. Joseph, personal communication). Therefore, it may be that NMID are natural components in certain lipids of marine crustacea.

Of the other fatty acids with potential for marine food chain research, the *trans*-6-hexadecenoic acid found in coelenterates (25,56), marine turtles (57), and the spadefish (58), is apparently inert as far as further participation in biochemical reaction is concerned. It may physically mimic 16:0, facilitating inclusion in depot fat triglycerides of the turtles. The precise origin of *trans*-6-hexadecenoic acid is as yet unknown, but the unusual 18:5ω3 is clearly of algal origin (59-61). As it disappears rapidly on passage up a marine food chain (16), it may be less useful in higher trophic levels than unusual mono- or diethylenic fatty acids such as the *trans*-6-hexadecenoic acid and the NMID. The 7-methyl-7-hexadecenoic acid and 7-methyl-6-hexadecenoic acid and homologues (58,62) are probably also inert. Although possibly at least in part of endogenous origin in higher animals, they do apparently accumulate from more primitive species.

Kochi has recently published papers reporting the isolation (63) and identification (64) of isomeric nonmethylene-interrupted eicosadienoic acids from the lipids of the sea urchin *Anthocidaris crassispina*. In addition to 5,11-eicosadienoic acid, accompanied by 6,11-eicosadienoic acid, a 3,11-eicosadienoic isomer is also described.

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Studies on Long Chain *Cis*- and *Trans*-acyl-CoA Esters and Acyl-CoA Dehydrogenase from Rat Heart Mitochondria[1]

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ABSTRACT

The β-oxidation of long chain fatty acids was investigated in a preparation of rat heart mitochondria. The acyl-CoA esters of the *cis* and *trans* isomers of Δ9-hexadecenoic, Δ9-octadecenoic, Δ11-eicosenoic, and Δ13-docosenoic acids were prepared. Rates of the acyl-CoA reaction were determined with an extract from rat heart mitochondria. The apparent Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated for each substrate. In general, apparent V_{max} values decreased with increasing chain length of the monoenoic substrates. Reduced activity of acyl-CoA dehydrogenase with long chain acyl-CoA esters could have contributed to accumulation of lipids in hearts of rats fed diets containing long chain fatty acids.

INTRODUCTION

Fat accumulated in the hearts of rats within a few days after diets containing long chain fatty acids were fed (1-7). The triglyceride fraction accounted for most of the accumulated fat (4,7). In in vitro preparations, rat heart mitochondria oxidized erucic acid more slowly than palmitic or oleic acids (8-14). Heijkenskjöld and Ernster reported lower maximum velocities for the acyl-CoA dehydrogenase reaction with erucyl-CoA than with palmityl-CoA for a beef heart mitochondria extract (12). To study this reaction further with more substrates, acyl-CoA esters of *cis* and *trans* isomers of Δ9-hexadecenoic, Δ9-octadecenoic, Δ11-eicosenoic, and Δ13-docosenoic acids were prepared and compared with palmityl-CoA.

EXPERIMENTAL PROCEDURES

Chemicals

All fatty acids (99+% pure) were purchased from Applied Science Labs, Inc., (State College, PA) except for the *trans* 20:1 isomer which was prepared by isomerization of the *cis* isomer (15) and purified by silver ion thin layer chromatography (TLC) of its methyl ester. Coenzyme A (free acid form, 91-96% pure) was purchased from P-L Biochemicals, Inc. (Milwaukee, WI). A stock solution in water (100 ml, 4 mg CoA/ml) was stored frozen for the duration of the experiments. Free sulfhydryl groups were unchanged throughout this period indicating that no oxidation had occurred (16). Tetrahydrofuran (THF) was freshly distilled over sodium for each preparation. For the acyl-CoA dehydrogenase assay, flavin adenine dinucleotide, phenazine methosulfate, bovine serum albumin fraction V, and 2,6-dichlorophenolindophenol were purchased from Sigma Chemical Co. (St. Louis, MO); n-hexadecanoyl-CoA was purchased from P-L Biochemicals, Inc.

Preparation of Acyl-CoA Esters

All acyl-CoA esters were prepared essentially by the modification of Seubert's procedure (17), described by Reitz et al. (18).

Gas Liquid Chromatography (GLC) Determination of Esterified Fatty Acid in Acyl-CoA Esters

An accurately known weight (~1 mg) of thiol ester with a known weight of heptadecanoic acid (0.25 mg) as internal standard was refluxed with 4 ml 0.5 *N* NaOH in MeOH for 15 min. Then 5 ml BF_3/MeOH were added through the condenser and the mixture refluxed a further 15 min. Esters were extracted with hexane and analyzed by GLC. Treatment of the thiol esters with diazomethane and GLC of the ether extract revealed insignificant amounts of nonesterified fatty acids present in the thiol esters.

Animals and Enzyme Preparation

One hundred and fifty-six male Sprague-Dawley rats weighing 45 to 70 g were purchased from BioBreeding Laboratories, Ottawa. They were fed a commercial cube diet (Master Fox Cubes) and water ad libitum for 2 wk. The rats weighed an average of 127 g when they were killed. Mitochondria were prepared from the hearts by the method described by Pande and Blanchaer (19). The mitochondrial pellet was suspended in 7.5 mM potassium buffer and then sonicated four times for 20 sec intervals with 50% pulsing using a Branson model 350

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TABLE I

Yields and Properties of Acyl-CoA Esters

Acyl group	Isomer	Yield[a] (%)	Molar extinction[b] at λmax 260, x 10^3	A_{232}/A_{260}[c]	Fatty acid in CoA ester (%)	
					Theoretical	Found
Δ9-hexadecenoic	*cis*	59	13.6	0.55	24.0	23.7
	trans	57	13.7	0.54	24.0	24.3
Δ9-octadecenoic	*cis*	75	13.2	0.55	26.0	27.3
	trans	46	13.1	0.54	26.0	25.0
Δ11-eicosenoic	*cis*	69	13.2	0.57	27.9	27.9
	trans	58	13.6	0.53	27.9	25.4
Δ13-docosenoic	*cis*	70	12.8	0.54	29.6	31.0
	trans	53	12.9	0.52	29.6	28.6

[a]Based on CoA, assuming molecular weight (MW) of 820.6 for CoA and MW of (820.6 + MW acid -18) for ester.

[b]Molar extinction of CoA was 14.2 x 10^3 at pH 7 in water.

[c]Ratio of absorbance at λmax 232 to that at λmax 260.

[d]Average of two determinations.

Sonifier. The mitochondrial suspension was centrifuged for 15 min at 30,000 x g. The supernatant was fractionated with solid ammonium sulfate. The 50 to 80% ammonium sulfate fraction was suspended in 40 mM potassium phosphate, pH 6.8, buffer; dialyzed against 40 mM potassium phosphate, pH 6.8, buffer for 18 hr; split into aliquots and then stored frozen. A new aliquot was thawed and used each day for the acyl-CoA dehydrogenase assay.

Acyl-CoA Dehydrogenase Assay

The assay used was a modification of the method described by Hoskins (20). The assay mixture contained potassium phosphate, 37.5 μmol, pH 6.8; 2,6-dichlorophenolindophenol, 12.4 nmol; flavin adenine dinucleotide, 43.8 nmol; phenazine methosulfate, 0.33 nmol; mitochondria extract, 9.0 μg protein [determined using the method described by Lowry et al. (21)]; 0 or 30 μg bovine serum albumin; and substrate 1.0, 1.2, 1.4, 1.8, 2.3, 3.3, 6.0, 10.0, or 30.0 nmol in a final volume of 0.3 ml. Assays were conducted in a Gilford, Model 240, recording spectrophotometer fitted with a Haake constant temperature circulating water bath set at 30 C. Each assay was run in triplicate with a single substrate blank. The phenazine methosulfate was protected from light. A premix was prepared containing everything except the phenazine methosulfate, substrate, and mitochondria extract. The phenazine methosulfate and mitochondria extract were added to the premix in the cuvette, then in about 2 min the substrate was also added. Reaction rates were linear with time for about 2 min. A molar extinction coefficient for 2,6-dichlorophenolindophenol of 19.7 x 10^3 mol^{-1} cm^{-1} was used (22).

To improve the interpretation of the kinetic data, and to obtain estimates of the reliability of the fitted constants, the nonlinear least-squares Fortran program described by Cleland was used to obtain estimates of the apparent Michaelis constant (K_m) and the maximum velocity (V_{max}) for each substrate (23). To determine whether chain length or configuration influenced these estimates, comparable groups of estimates were analyzed by a weighted least squares regression routine. Each estimate was weighted by the reciprocal of its estimated variance; hence, appropriate tests could be performed on the basis that the resulting regression sums of squares were distributed as approximately X^2. A pooled variance estimate was not used in these latter analyses since the variances were judged by Bartlett's test to be unequal. Finally, a limited number of comparisons between pairs of estimates were performed using an approximate t-test (23).

RESULTS AND DISCUSSION

Acyl-CoA Esters

The yields and some properties of the acyl-CoA esters are summarized in Table I.

Under the reaction conditions adopted, the pH reading, measured with a standard combination electrode and digital pH meter, had to be held at 8.8 to 9.0 for reaction to occur. When this reading was held at 7.5-8.0 (17,18,24), yields were poor. It is probable that under the predominantly nonaqueous conditions (THF:H_2, 7:3) the true pH was not being recorded. Yields of thiol esters ranged from 46-75% and seemed independent of the chain length of the acyl group. However, yields from

TABLE II

Acyl CoA Dehydrogenase Activity[a] with Each Acyl-CoA Ester

Acyl group	Isomer	Albumin[b] (μg/assay)	Substrate concentration (μmolar) 3.4	4.0	4.8	5.9	7.7	11.1	20.0	33.3	100
n-hexadecanoic	-	0	2.25[a]	2.48	3.42	3.58	4.34	4.26	5.16	6.08	6.08
		30	2.08	2.32	2.59	2.94	3.58	3.90	5.28	5.46	6.05
Δ9-hexadecenoic	*cis*	0	3.01	3.39	3.20	3.54	3.81	4.38	4.80	5.41	5.18
		30	2.32	2.55	2.57	2.89	3.29	4.21	5.12	5.69	6.09
	trans	0	3.16	3.58	4.42	4.53	4.61	5.90	5.79	6.19	6.21
		30	3.37	3.41	3.68	4.36	5.16	5.22	5.92	7.01	6.63
Δ9-octadecenoic	*cis*	0	2.63	3.39	3.98	3.46	3.96	3.73	4.72	4.66	4.82
		30	1.77	2.17	2.55	2.46	2.99	3.71	4.68	4.53	5.01
	trans	0	3.05	3.58	3.52	3.98	4.26	4.51	5.20	5.26	5.92
		30	2.66	2.89	3.41	3.64	4.08	4.55	5.27	5.39	5.90
Δ11-eicosenoic	*cis*	0	1.43	1.94	2.36	2.17	2.63	2.89	3.03	3.73	3.62
		30	1.01	1.03	-[c]	1.66	1.98	2.49	2.78	2.86	4.06
	trans	0	1.47	1.24	1.30	1.50	1.05	1.79	2.10	2.23	2.42
		30	0.48	0.61	0.57	0.94	0.50	1.58	1.85	2.28	2.44
Δ13-docosenoic	*cis*	0	0.76	1.01	1.05	1.51	1.68	1.79	1.88	2.17	2.24
		30	0.25	0.44	0.77	0.82	1.12	0.99	1.10	1.47	2.28
	trans	0	0.74	0.70	0.93	0.91	1.24	1.16	1.52	1.41	1.81
		30	0.32	0.55	0.46	0.63	0.84	0.99	1.28	1.18	2.17

[a]Nanomoles 2,6-dichlorophenolindophenol reduced per minute per assay X10 in a volume of 0.3 ml. Each value was the average of three determinations.
[b]Bovine serum albumin fraction V.
[c]Missing value.

TABLE III

Apparent Michaelis Constant (K_m) and Maximum Velocity (V_{max}) for Each Acyl-CoA Ester[a]

Acyl group	Isomer	K_m			V_{max}		
		Value[b]	S.E.[c]	S.E.[c]/K_m (%)	Value[d]	S.E.[c]	S.E.[c]/K_m (%)
n-hexadecanoic	-	5.4	0.44	8	6.7	0.17	3
Δ9-hexadecenoic	*cis*	3.0	0.22	7	5.5	0.10	2
	trans	3.0	0.28	9	6.7	0.10	2
Δ9-octadecenoic	*cis*	2.2	0.30	14	5.0	0.15	3
	trans	3.1	0.15	5	5.9	0.08	1
Δ11-eicosenoic	*cis*	4.2	0.46	11	3.9	0.12	3
	trans	3.5	0.34	10	2.5	0.06	3
Δ13-docosenoic	*cis*	5.5	0.57	10	2.5	0.08	3
	trans	5.2	0.59	11	1.8	0.06	4

[a]Estimated from the 0 μg/assay albumin data, Table II. See text.

[b]μmolar.

[c]Standard error.

[d]Nanomoles 2,6-dichlorophenolindophenol reduced per minute per assay X10, in a volume of 0.3 ml.

the *trans* 18, 20, and 22 acids did appear lower than those from the corresponding *cis* isomers.

Spectral analysis of the thiol esters (pH 7) indicated that the ratios of absorbance at 232 nm to that at 260 nm ranged from 0.52-0.57, which is characteristic of acyl-CoA esters (17,25). From a comparison of molar extinctions for the thiol esters to those obtained from CoA itself (14.2×10^3), the acyl-CoA derivatives would appear to range in purity from 90.1-96.4%, with the purity tending to decrease as the acyl chain length increased. This assumes that acyl groups do not affect the absorbency of the CoA derivatives which may in fact not be the case.

From the GLC data, except for the *trans* 20:1-CoA ester, all fatty acids bound as esters were within 5% of theoretical values. This procedure for ester determination was selected over the more common spectroscopic hydroxamate test (26,27) because of solubility problems encountered with the 20 and 22 *trans* fatty acid derivatives. It is interesting to note that acid-catalyzed methanolysis (2% H_2SO_4: MeOH, 1 hr reflux) produced only 25% transesterification of these thiol esters.

TLC on cellulose using BuOH:HOAc:H_2O 5:2:3 as solvent (28) showed the major UV absorbing spot, with the 16 and 18 thiol esters, to be at Rf 0.8. With the 20 and 22 thiol esters, a streak was observed ranging from Rf 0.6-0.8. All possessed a minor UV absorbing spot at Rf 0.2. Neither CoA nor oxidized CoA was observed in any sample except for a trace of oxidized CoA in *trans* 20:1-CoA ester.

Acyl-CoA Dehydrogenase

Albumin was used in the butyryl-CoA dehydrogenase assay described by Hoskins (20). The assay was linear for longer time periods when albumin was used in the assay mixture. However, at low substrate concentrations acyl-CoA dehydrogenase activities tended to be lower with albumin (Table II). These lower activities could likely be attributed to albumin binding acyl-CoA and, therefore, reducing the effect substrate concentration (14). For that reason, the 30 μg albumin/assay data were not used in the estimation of apparent K_m and V_{max} values (Table III).

The kinetic data are presented in Table III in which separate columns indicate the standard error for each estimate, as well as the standard error expressed as a percentage of the quantity estimated. In experiments of this type, percentages of 10% or less indicate a good fit, while percentages in excess of 25% are indicative of a poor fit (23). The fits for V_{max} were very good and for K_m were fairly good. The apparent K_m for n-hexadecanoyl-CoA was higher ($P<0.05$) than the values for *cis*- or *trans*-Δ9-hexadecenoyl-CoA. The V_{max} values were similar for n-hexadecanoyl-CoA and *trans*-Δ9-hexadecenoyl-CoA. In general, for the monoenoic substrates, apparent K_m values increased and V_{max} values decreased with increasing chain length ($P<0.001$). The relative relationship between the V_{max} values for n-hexadecanoyl-CoA and *cis*-Δ13-docosenoyl-CoA was consistent with that reported by Heijkenskjöld and Ernster for a beef heart mitochondria extract (12). They reported a relatively lower value for *cis*-Δ9-octadecenoyl-CoA. They reported one K_m (4.6 μmolar) for all three substrates, and therefore obtained a relatively high value for *cis*-Δ9-octadecenoyl-CoA.

As discussed by Swarthouw, the mitochondrial oxidation of a long chain fatty acid may be reduced at the stage of activiation, transfer across the inner mitochondria membrane or β-oxidation (14). At each step, he found that the reaction was slower with erucic acid than with palmitic acid. An obvious point of control on the basis of chain length would be the acyl-CoA dehydrogenase system since there are at least three different dehydrogenases, one of which has been postulated to possess activity with substrates up to about C_{22} (29,30). Bunyan and Greenbaum (31) have suggested that the acyl-CoA dehydrogenase reaction was rate limiting for β-oxidation in rat liver. The observed activities of the acyl-CoA dehydrogenase system strongly suggest that it is an important control point in fatty acid metabolism. Reduced activity of acyl-CoA dehydrogenase with long chain acyl-CoA esters could have contributed to accumulation of lipid in hearts of rats fed diets containing long chain fatty acids.

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The Nonpolar Egg Wax Lipids of the Cattle Tick, *Boophilus Microplus* (Canestrini)

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ABSTRACT

About 40% of the lipids of the egg wax of *Boophilus microplus* are nonpolar and comprise hydrocarbons (10%) and wax esters (90%). The hydrocarbon fraction is 87% unbranched alkanes, the major components being n-nonadecane (10.8%), n-eicosane (12.1%), and n-heneicosane (9.8%). This fraction also contains 2,4,6-cholestatriene (4.6%). The majority of the acids are branched (77%), the major components being 12-methyltetradecanoic (16.3%) and branched hexadecanoic acids (28%). The major alcohols are n26:0 (20.4%), n28:0 (47.3%) and n36:0 (18.2%). A steroid, possibly 2,4,6-cholestatrien-25-ol (5.6%), is also present.

INTRODUCTION

The cattle tick, *Boophilus microplus*, has for almost a century been a major economic threat to the Australian cattle industry, a threat exacerbated by the increasing occurrence of chemically resistant strains (1-3) as well as by the proliferating restrictions on chemical controls imposed by public health bodies.

As an egg this arthropod would seem most vulnerable, being most exposed to predation and most subjected to environmental hostility. Desiccation of the eggs occurs at low humidity (4) and superficial waxes are significant in the water economy of arthropods (5). However, most chemical studies of surface wax constituents have been effected on insect cuticle waxes: with the exception of the wax esters of the coccid, *Lecanium horii*, (6) there are virtually no composition data on egg waxes as such. The *cri de coeur* for "quantitative chemical data . . . obtained by modern analytical techniques" (7) reflects not only the biological significance of the waxes but also their chemical complexity.

Kitaoka (8) observed a rather constant transfer of steroid material to the eggs but found an unexpectedly small amount of sterol in ticks which had completed oviposition. Current investigations of the sterol economy (9) reveal that 49-63% of the cholesterol inherent in the engorged tick is unaccounted for by lipoid in the spent female and her eggs. The balance, unchanged or converted, must be present in the egg wax or lost as water soluble metabolites.

Unlike other arthropods whose eggs achieve impermeability before they are laid by either a waterproofing deposition or an impregnation on the inside of the shell (10), ticks seem unique in that they elaborate the impermeability of their eggs by an external investment of wax, applied subsequent to oviposition by a specialized gland, Gené's Organ, which has no counterpart in other acarina (11).

In order to determine the chemical role of Gené's Organ, we initiated an investigation of the egg wax constituents. This paper reports the identity of the nonpolar lipids.

EXPERIMENTAL PROCEDURES

All solvents used were redistilled through 60 cm columns packed with glass helices. Woelm neutral alumina, activity III, was used for column chromatography; Merck prepared plates for thin layer chromatography (TLC). Visualization was effected with iodine vapor, 0.5% aqueous potassium permanganate, or 1% vanillin in 50% aqueous phosphoric acid. Gas liquid chromatography (GLC) was carried out on a Hewlett Packard 5710A instrument using 6 ft stainless steel columns; stationary phases and temperatures are indicated in the text. UV spectra were recorded on a Unicam SP800A spectrophotometer, mass spectra and accurate mass measurements on an AEI MS902S mass spectrometer, and combined gas chromatography-mass spectrometry (GC-MS) on a Varian MAT111 instrument.

A typical extraction procedure was as follows. Tick eggs (100 g) were washed several times with hexane, care being taken to prevent any rupture of the membrane. The hexane washings were centrifuged at 5000 g (5-10 min), combined and evaporated under vacuum at room temperature, and yielded a creamy-yellow waxy solid (436 mg). The solid was chromatographed on alumina (30 g) eluting with hexane (100 ml). Evaporation of the solvent as above gave a waxy residue (17.5 mg

TABLE I

Composition of Hydrocarbons

Compound number	ECL[a]	Percentage composition[b]	Structure	Identification[c]
1	13.0	0.8	n-C_{13}	G.M.
2	13.15	0.6		
3	14.0	1.7	n-C_{14}	G.M.
4	14.5	0.3		
5	15.0	1.3	n-C_{15}	G.M.
6	16.0	3.5	n-C_{16}	G.M.
7	16.3	0.7		
8	16.85	1.5		
9	17.0	5.5	n-C_{17}	G.M.
10	19.0	10.8	n-C_{19}	G.M.
11	20.0	12.1	n-C_{20}	G.M.
12	21.0	9.8	n-C_{21}	G.M.
13	22.0	8.8	n-C_{22}	G.M.
14	23.0	3.4	n-C_{23}	G.M.
15	24.0	3.4	n-C_{24}	G.M.
16	24.4	1.3		
17	25.0	3.8	n-C_{25}	G.M.
18	26.0	4.8	n-C_{26}	G.M.
19	27.0	4.6	n-C_{27}	G.M.
20	27.8	1.3		
21	28.0	1.7	n-C_{28}	G.M.
22	28.4	4.6	Cholestatriene	G.M.
23	29.0	1.5	n-C_{29}	G.M.
24	30.0	1.4	n-C_{30}	G.M.
25	31.0	1.4	n-C_{31}	G.M.
26	32.0	0.6	n-C_{32}	G.M.

[a]Equivalent chain length obtained from several isothermal semilog plots.

[b]Hydrocarbons below 0.3% not shown. The majority lie on three log plots of fractional chain length, 0.3, 0.5, and 0.7. See text.

[c]Structure determination. G = Retention time, M = Mass spectra.

4%) of hydrocarbons. Further elution with hexane:benzene (9:1, 50 ml) and vacuum evaporation of the solvent at room temperature afforded a white waxy solid (156 mg 36%) containing esters.

RESULTS AND DISCUSSION

Hydrocarbons

The hydrocarbon fraction could be separated by preparative TLC into saturated and unsaturated species. The latter comprised one component (GLC, SE30 column, 220 C) whose UV spectrum (cyclohexane) showed maxima at 295, 305, and 316 nm characteristic of steroid 2,4,6-trienes (12). Mass spectral analysis and catalytic hydrogenation (H_2/Pd in ethanol) to a mixture of 5α- and 5β- cholestane, whose GLC retention times and mass spectra were coincident with those of authentic samples, confirmed the structure published by Atkinson and Binnington (13). Though these authors found this to be the major hydrocarbon, our estimates based on gas chromatography show it to represent only 5% of the hydrocarbon fraction.

Gas chromatography (SE30 column, series of isothermal runs 120-220 C) of the saturated hydrocarbons indicated a complex mixture of over sixty compounds, 87% of which were unbranched C_{13} to C_{32} alkanes as shown by their retention times compared with standards, and mass spectra (GC-MS). The major components were n-nonadecane (10.8%), n-eicosane (12.1%), and n-heneicosane (9.8%). Both odd (49%) and even (51%) numbered chains occurred to the same extent. The results are detailed in Table I.

The gas chromatogram reveals a myriad of branched saturated hydrocarbons which represent 8% of the total hydrocarbon fraction. Semilog plots show these to lie on a series of straight lines, the major series having fractional chain lengths (FCL) of 0.3, 0.5, and 0.7. Admixture with authentic 2-methyl-hexadecane (*iso*heptadecane) and 3-methylpentadecane (*anteiso*hexadecane) [Analabs Inc., New Haven, CT], indicate the absence of *iso*alkanes but identify (on the basis of retention time) the 0.7 series as *anteiso*alkanes.

Esters

The mass spectrum of the crude ester frac-

TABLE II

Composition of Alcohols from Hydrolyzed Wax Esters

Compound number	Number of carbons	% Composition	Compound
1	26	20.4	n-hexacosanol
2	27	5.6	n-heptacosanol
3	28	47.3	n-octacosanol
4	30	2.0	n-triacontanol
5	32	0.4	n-dotriacontanol
6	34	3.0	n-tetratriacontanol
7	35	0.6	n-pentatriacontanol
8	36	18.2	n-hexatriacontanol
9	38	1.6	n-octatriacontanol
10	40	0.5	n-tetracontanol

tion indicated a complex mixture of aliphatic esters as shown by several series of peaks corresponding to structures $RCO_2R_1^{+\cdot}$, $RCO_2H_2^+$, $R_1OC(OH)=CH_2^{+\cdot}$, and $(R_1-H)^{+\cdot}$ ($R=C_{13}$-C_{18}, $R_1=C_{26}$-C_{28}) as shown by high resolution mass measurement. In addition, there were prominent peaks at m/e 364 $(C_{27}H_{40})^{+\cdot}$, 368 $(C_{27}H_{44})^{+\cdot}$, and 396 $(C_{29}H_{48})^{+\cdot}$ which could correspond to the sterols $C_{27}H_{42}O$, $C_{27}H_{46}O$, and $C_{29}H_{50}O$.

The UV spectrum of the crude ester fraction was superimposable on that of the triene, indicating the presence of an ester of a steroid trienol. Such a sterol, with seven degrees of unsaturation (rings plus double bonds) would appear as a homologue of a saturated alcohol and not be detected in the low resolution mass spectrum. However, high resolution measurements of m/e 364 showed it to be a doublet, $C_{26}H_{52}^{+\cdot}$ and $C_{27}H_{40}^{+\cdot}$.

The peak at m/e 368 is characteristic of cholesteryl esters though none could be detected by TLC. This observation is in direct conflict with the report (13) that the surface lipids contain mainly hydrocarbons and cholesteryl esters. The origin of the peak at m/e 396 is unknown, although $C_{29}H_{48}^{+\cdot}$ ($=C_{29}H_{50}O$) could correspond to sitosterol.

In order that a detailed analysis of the constituent fatty acids and alcohols could be carried out, the esters were saponified and the alkaline solution extracted with hot chloroform to isolate the alcohols which were acetylated. Acidification and extraction with hexane yielded the fatty acids which were converted to their methyl esters.

Alcohols

Preparative TLC of the alcohol fraction separated the sterol from the saturated alcohols. The UV spectrum confirms the chromophore (12) and other evidence (14) suggests that the compound is 2,4,6-cholestatrien-25-ol. Calculations based on the known molar extinction coefficient at 306 nm (14,500) indicate that the sterol represents 5.6% of the total alcohol fraction.

Gas chromatography of the alcohols (SE30, 220 C, and a 20 in. column of UCC-W982 at 320 C) produced a series of peaks whose semilog plot gave a straight line. GC-MS (OV17, 220-270 C, Δ4/min) of the alcohols and their acetates positively identified the compounds as n-alcohols. The composition of the mixture is given in Table II.

Methyl Esters

The gas chromatogram (SE30, 180 C) of the fatty acid methyl esters revealed a multicomponent mixture. Doping with authentic standards established the series of n-C_{14}–n-C_{19} acid methyl esters and enabled the equivalent chain lengths (ECL) of the other components to be calculated. The structures of these components were established by GC-MS (OV17, 120-300 C, Δ4/min) using established interpretation techniques (15). Several of the GC peaks were shown to be unresolved and their components were identified by recording mass spectra at several points through the single GC peak. All of the monomethyl acids could thus be subject to unambiguous structural assignment. These are shown in Table III.

Three compounds of the mixture (Nos. 15, 17, 18) were unresolvable and their structures are at best tentative. In an attempt to estimate the substitution sites from the ECLs of these compounds, the separation factors on a silicone column of the methyl esters of all the monomethyloctadecanoates (16) were converted into fractional chain lengths (FCL) by comparison with a semilog plot generated from our data ($r_{19:0}$ as 1.000, $r_{16:0}$ as 0.314, SE30, 180 C). The values obtained gave excellent agreement with the esters containing only one methyl branch, but for multiple branched esters the calculated ECL values were always higher than

TABLE III

Composition of Methyl Esters

Compound number	GLC peak number	ECL[a]	Number of carbons	% Composition[c]	Compound (as carboxylic acid)
1	1	14.0	14	5.1	tetradecanoic acid
2	2	14.5	15	5.7	8-methyltetradecanoic acid
3		14.5	15		10-methyltetradecanoic acid
4	3	14.8	15	16.3	12-methyltetradecanoic acid
5	4	15	15	0.8	pentadecanoic acid
6	5	15.18	16	3.3	6,12-dimethyltetradecanoic acid
7	6	15.5	16	5.4	8-methylpentadecanoic acid
8		15.5	16		10-methylpentadecanoic acid
9		15.5	16		11-methylpentadecanoic acid
10	7	15.65	16	1.0	12-methylpentadecanoic acid
11	8	16.0	16	11.2	hexadecanoic acid
12		16.5	17		8-methylhexadecanoic acid
13	9	16.5	17	28[d]	10-methylhexadecanoic acid
14		16.5	17		12-methylhexadecanoic acid
15		16.5	18	3[d]	?
16	10	16.8	17		14-methylhexadecanoic acid
17		16.8	18	11.1	9,11-dimethylhexadecanoic acid[b] (?)
18		16.8	18		10,12-dimethylhexadecanoic acid[b] (?)
19	11	17.0	17	1.8	heptadecanoic acid
20	12	17.18	18	2.9	8,14-dimethylhexadecanoic acid
21	13	17.5	18	1.9	8-methylheptadecanoic acid
22		17.5	18		10-methylheptadecanoic acid
23	14	18.0	18	3.7	octadecanoic acid
24	15	18.5	19	1.8	8-methyloctadecanoic acid
25		18.5	19		10-methyloctadecanoic acid
26		19.0	19	trace	nonadecanoic acid

[a]Generated from semilog plot of unbranched esters, $r_{18:0}$ as 1.00, $r_{16:0}$ as 0.462.

[b]Tentative only; see text.

[c]Calculated from integration of GC peaks. Thus, e.g., Numbers 7, 8, and 9 = 5.4%.

[d]Estimated from molecular ion relative intensities.

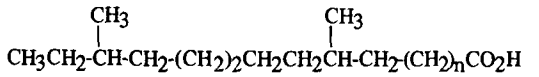
the observed ones. The failure of apolar columns to yield additive FCL values has been previously noted (17). From these, however, it would appear that compound No. 15 is at least trisubstituted.

Unlike the hydrocarbons, only a small percentage of the fatty acids is unbranched (22.6%). Two anteiso acids both containing an odd number of carbon atoms are major constituents (27%). Multiple branched acids are present in small amounts (ca. 5%) while most of the acids (45%) have branches in the middle of the chain. Some 65% of the total acids have an odd number of carbons with C_{17} predominating.

Esters comprise 36% of the total egg wax and 90% of the nonpolar lipids, hydrocarbons the other 10%. The average calculated molecular weight is 610, remarkably close to Gilby's estimate (18) for tick cuticle wax of 600, based on infrared spectroscopy and monolayer film techniques. He concluded that the wax was composed of hydrocarbons and esters of long chain acids and alcohols but did not identify any of the constituents.

The nature of these constitutents deserves some comment. Most data quoted for arthropods are for cuticle wax acids and these cite carbon numbers and degree of unsaturation only and do not lend themselves, therefore, for comparison. However, medium chain length acids with methyl branches in any of the 8-, 10-, or 12-positions occur frequently in nature. They are generated as a waterproofing agent by the preen glands of the swan, *Cygnus olor* (19), and are found in dairy products (20) and ruminant tissue (21). The esterifying C_{26} and C_{28} alcohols are common in hair lipids (22) and wool wax (23) and would seem to be general superficial lipoid material. The tick waxes may, therefore, in part reflect the dietary origins of the tick, and their deposition represents a means of utilization.

The two dimethyl-carboxylic acids (compounds 6 and 20, Table III) have not previously been isolated. Both are anteiso acids. Biogenetic theories (24) associate anteiso acid formation with a mechanism initiated by isoleucine. These molecules have the general formula:



(n = 3,5)

incorporating two isoleucine skeletons. Whatever the detail, it is obvious two biogenetic pathways are involved.

No unsaturated acids were detected. The egg waxes differ considerably, therefore, in composition from the cuticle waxes of, e.g., the bull ant, *Myrmecia gulosa*, (75% oleic acid) (25), and the mealworm, *Tenebrio molitor* (20% oleic acid) (26), both in their saturation and the high percentage of branched chain acids.

The overall percentage of hydrocarbons is low, whereas in insect cuticle waxes, it appears much higher (24). There is no preference for odd or even numbered carbon chains and only a small percentage of the hydrocarbons, unlike the acids, suffer branched chains. As in the acids however, unsaturation is absent. This may reflect the semitropical habitat of the cattle tick and the nature of its microenvironment on the host as it is well known that unsaturation increases the mobility of waxes and is, therefore, more likely to be encountered on cuticles frequenting more temperate environments.

The surface nonpolar lipids of tick egg wax insofar as they contain hydrocarbons and esters bear some resemblance to the cuticle waxes of insects that have been investigated. The biological role of cuticle waxes for cattle ticks has been suggested to include waterproofing, protection from the physicochemical hazards of the environment, and a repository of nonutilizable lipid (27). Tick egg waxes may satisfy these requirements but, in addition, may well serve other functions. This suggestion is strengthened by a comparison with the waxes of the coccid, *L. horii* (6), which are comparatively simple and contain almost exclusively simple wax esters. Thus, they would seem to fulfill the simple task of controlling the water economy of the eggs. The complexity of the tick egg wax intimates other, more subtle mechanisms.

The role of the steroids in the egg wax is unknown though it is unlikely that they contribute merely to the waterproofing of the egg. This can be achieved by chemicals far less complex to which the tick has access. Besides, the 2,4,6-triene structure of the steroids is readily oxidized in the absence of porose tissue secretion (13) which renders them unlikely compounds for such a task. The steroids are absent in the egg tissue and the cuticle secretions of the engorged tick but appear in extracts of cuticle waxes of ovipositing ticks, though whether this is due to a general secretion or merely to a spread from Gené's organ is not known. It is known, however, that once oviposition is under way, a film of the secretion from Gené's organ spreads over the legs and cuticle (11), and this is the more probable source of the steroids in the cuticle wax at this time as it would seem uneconomical and unnecessary for the tick to change the compo-

sition of her cuticle wax at this terminal stage in her life cycle. It is possible that these steroids though externally deposited in the wax may well be able to penetrate the egg membrane and satisfy some as yet unknown embryonic requirement.

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Ether-Linked Glycerolipids in Human Brain Tumors

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ABSTRACT

In this investigation, the lipid composition of a number of human brain tumors was determined and compared to that of normal adult brain. Glioblastomas (11 samples), astrocytomas (4 samples), an acoustic neurinoma, an oligodendroglioma, and a meningioma were analyzed. All of the tumors had substantial levels (0.8-3.4% of total phospholipids) of choline plasmalogen which was present in only trace amounts in normal brain. With the exceptions of the acoustic neurinoma and the meningioma, the concentration of alkylacylglycerophosphorylcholine was also higher in the tumors than in normal brain. Neutral lipids of brain tumors also contained high concentrations of both alkyl (1.6-4.8% of total neutral glycerolipids) and alkenyl diacyl glycerol (3.8-10.1%). The results from this investigation indicate that increases in ether-linked glycerolipids may be characteristic of human brain tumors.

INTRODUCTION

The purpose of this investigation was tc compare the content of ether-linked glycerolipids of the phospholipids and neutral lipids of different classes of human brain tumors with that of normal human brain. Interest in ether-linked glycerolipids and their possible function in neoplastic tissue was initially generated by the findings of Wood and Snyder which established that a variety of animal and human tumors contained high concentrations of ether-linked glycerolipids (1,2). Other investigators have subsequently found similar high levels in other neoplastic tissue, including transplantable hepatomas and tumor cells grown in tissue culture (3-5). Accordingly, Snyder (6) has proposed that high levels of alkyl glycerolipids in tissue can be used as a marker for malignant tumors. Howard et al. (5) found that in a series of transplantable hepatomas and in hepatomas grown in cell culture there is a correlation between an increase in growth rate and an increase in ether-linked glycerolipids. These observations led the authors to suggest that the increased content of ether-linked glycerolipids in tumors may be related to differentiation and adaptation associated with an increased capacity for tumor growth.

Most of the composition studies of ether-linked lipids of tumors have been conducted on tumors of nonneural origin and relatively little information is available on the ether-linked lipid composition of nervous tissue tumors such as brain tumors. Analyses that have been reported generally have been limited to the determination of plasmalogens. Slagel et al. (7) observed a relatively low level of ethanolamine plasmalogen in glioblastomas, whereas Sun and Leung (8) found an elevated level of this lipid in these tumors. Neither the alkyl acyl glycerophospholipids nor the content of the ether-linked neutral lipids was determined in the work by Sun and Leung. In light of the possible significance of ether-linked lipids in the development of neoplastic tissue, this investigation was undertaken to determine if alterations in the content of ether-linked lipids are characteristic of tumors from human brain, a tissue which normally contains a high concentration of ether-linked lipids, primarily as ethanolamine plasmalogens (9).

MATERIALS AND METHODS

Human brain tumor samples were obtained during surgical procedures from Columbia University, the Medical University of South Carolina, and the Medical School of the University of North Carolina at Chapel Hill. The type of tumor represented by each sample was diagnosed by the laboratory furnishing the sample. The samples were kept in ice-cold saline solution until extraction procedures were initiated, generally within 1 hr after surgical removal. Normal brain specimens were obtained during autopsy within 12 hr after death. These tissues, if not analyzed immediately, were stored frozen. Gray matter was obtained from the brain samples by removing all visible traces of white matter.

1-Dodecyl glycerol for use as an internal standard in the determination of the content of alkyl ether was obtained from Supelco, Inc. (Bellefonte, PA). The internal standard used for measurement of the content of alkenyl ether, 1-heptadec-1′-enylglycerol, was chemically synthesized by the method of Gigg and Gigg (10). Heptadecanal, required as a starting

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product, was prepared by oxidation of the methanesulfonate of 1-heptadecanol (Eastman Kodak Co., Rochester, NY) with dimethyl sulfoxide as described by Mahadevan et al. (11). The purity of the final product was demonstrated by thin layer chromatography (TLC) in a developing solvent of diethyl ether:acetic acid 100:05 v/v by comparison with alkenyl glycerols obtained from natural sources. The presence of a vinyl ether bond was verified by obtaining a positive reaction with dinitrophenylhydrazine reagent (12). Further verification of the structure of the synthesized standard was obtained by subjecting the standard to acid hydrolysis and analyzing the released free aldehyde by gas liquid chromatography (GLC). Hydrogenation of the vinyl ether double bond resulted in the formation of alkyl glycerol as was demonstrated by TLC and GLC of the isopropylidine derivative. The infrared spectrum of a chloroform solution of the standard was also obtained and this compared closely with published spectra of alkenyl glycerols of other chain lengths (10,13).

Other lipid standards and Silica Gel G and H were purchased from Supelco, Inc., Bellefonte, PA. Grand Island Biological Company, Grand Island, NY, supplied the phospholipase C from *Bacillus cereus*.

Total lipids were extracted from a 0.5-1.0 g sample of brain tumor with chloroform: methanol 2:1 v/v and washed by the procedure of Folch et al. (14). Neutral lipids were separated from phospholipids on a short column containing 1 g silicic acid. Neutral lipids were eluted with 50 ml of chloroform and phospholipids with 50 ml methanol. Triacyl glycerol, alkyl diacyl glycerol, and alkenyl diacyl glycerol were obtained from the neutral lipid fraction by preparative TLC using a developing solvent of hexane:diethyl ether:acetic acid 60:40:1 v/v (10). The method described by Dittmer and Wells (15) based upon the infrared absorbance of esters was used to estimate the amount of triacyl glycerol. Triolein was used as a standard.

The phospholipids were separated into the major phospholipid classes by TLC on Silica Gel H with chloroform:methanol:acetic acid: water 50:25:8:4 v/v (16). Visualization was accomplished by spraying with a 0.05% water solution of Rhodamine-6G and viewing under a UV light source. The phospholipids were eluted from the silica gel scrapings by successive washes with methanol, chloroform:methanol 1:9 v/v, chloroform:methanol 2:1 v/v, and chloroform. Lipid phosphorus was determined by the method of Bartlett (17). Aliquots of the separated phospholipid classes were analyzed by analytical TLC with sulfuric acid charring. Fractions containing more than one spot on the test plate were rechromatographed.

The method for the analysis of ether-linked lipids was based upon quantitation by GLC with an internal standard and involves a stepwise degradation of intact phospholipids. The purified phospholipids were subjected to hydrolysis by phospholipase C essentially as described by Waku et al. (18) under conditions which assured quantitative removal of the phospho-base moiety from the diacyl and ether-linked phospholipids (19). The resulting diacyl glycerol, and alkyl and alkenyl acyl glycerols were separated by preparative TLC (hexane: diethyl ether:acetic acid 60:40:1 v/v). Known amounts of 1-heptadec-1′-enyl glycerol and 1-dodecyl glycerol were added as internal standards to the band of silica gel containing the alkyl and alkenyl acyl glycerols, which was then subjected to mild alkaline hydrolysis. The resulting free fatty acids, alkenyl glycerol, and alkyl glycerol were resolved by preparative TLC (diethyl ether:acetic acid 100:0.5 v/v). Aldehydes were prepared from alkenyl groups by acid hydrolysis (20) and isopropylidene derivatives of alkyl glycerols were prepared by the procedure of Wood (21). These derivatives were separated by GLC on a 12 ft x 0.08 in. coiled glass column packed with 12% EGSS-Y on 100-200 mesh Gas Chrom Q (Applied Science Labs, Inc.). The instrument was a Perkin-Elmer Model 900 equipped with dual hydrogen flame ionization detectors and a digital integrator. The column temperature was 195 C for the isopropylidene derivatives and 170 C for the aldehydes. Injection port and manifold temperatures were 230 C and 250 C, respectively. Peak areas were corrected for differences in molecular weight by multiplying the peak area by the molecular weight ratios of the naturally occurring moiety (C_{16}, C_{18}, $C_{18:1}$) and the internal standard. Total moles of aldehyde or isopropylidene was calculated by comparing the sum of the corrected peak areas of the naturally occurring moieties with the corrected area of the internal standard.

The sensitivity and operating range of the GLC portions of the method were determined by analyzing a series of samples containing varying amounts of methyl stearate and heptadec-1′-enyl glycerol or 1-dodecyl glycerol. The response was linear for both internal standards over a range of 0.04 μg to 5.00 μg of sample per injection.

As a further check of the method for the quantitation of alkenyl moieties, the ethanolamine phosphoglycerides from lipids of mature rat brain were analyzed by the above proce-

TABLE I

Phospholipid Class Composition of Normal Human Brain Gray Matter and Human Brain Tumors[a]

Phospholipid[b]	Normal adult gray matter	Glioblastomas	Astrocytomas	Acoustic neurinoma	Oligodendroglioma	Meningioma
	[7][c]	[11][c]	[4][c]	[1][c]	[1][c]	[1][c]
Sphingomyelin	10.4 (0.3)	13.6 (0.8)	12.8 (1.2)	16.1	14.3	16.1
Inositol and serine phosphoglycerides	12.7 (0.6)	14.4 (0.7)	18.3 (1.4)	12.0	10.3	23.5
Diacyl GPE	30.5 (0.5)	12.1 (1.1)	19.2 (4.3)	20.8	15.7	16.2
Alkyl acyl GPE	0.4 (0.1)	0.6 (0.1)	0.5 (0.2)	0.3	1.2	0.3
Alkenyl acyl GPE	4.9 (0.9)	8.3 (1.9)	6.2 (1.1)	0.8	3.1	11.8
Diacyl GPC	36.7 (0.6)	42.9 (0.8)	30.3 (1.1)	45.6	41.6	33.7
Alkyl acyl GPC	0.5 (0.1)	2.0 (0.6)	2.7 (1.0)	0.3	1.2	0.4
Alkenyl acyl GPC	0.1 (0.1)	1.7 (0.5)	2.4 (1.0)	0.8	3.1	3.4
Total ether-linked phospholipids	5.9	12.6	11.8	1.2	8.6	15.9

[a]Data expressed as percent of total lipid phosphorus. Numbers in parenthesis are the standard error.
[b]GPC = glycerophosphorylcholine; GPE = glycerophosphorylethanolamine.
[c]Number of individual samples analyzed.

dure. The plasmalogen content was found to be 69% of the phosphatidyl ethanolamine fraction or 26% of the total phospholipids. The latter value is in close agreement with that previously reported by other investigators (21.9% of total phospholipid, ref. 22).

RESULTS AND DISCUSSION

The phospholipid composition of normal human brain gray matter was obtained for comparison with that of the human brain tumors. This comparison is not entirely satisfactory since gray matter contains many different cell types (23). However, gray matter was considered to afford a better comparison than whole brain tissue or white matter which contains a high proportion of myelin (23) not present in tumors. With the exception of the ethanolamine plasmalogen content, the phospholipid composition of normal human brain gray matter in Table I is in good agreement with compositional data reported by other authors (24). The relative amount of ethanolamine plasmalogen reported in Table I, however, is substantially lower than the concentration usually reported (12 to 16% of total phospholipids of human gray matter, ref. 25). This discrepancy may result in part from possible differences in the proportion of contaminating white matter in the samples analyzed. White matter consists of 30-40% myelin which has a high concentration of ethanolamine plasmalogen (30-50% of total phospholipids, ref. 25). The higher content of ethanolamine plasmalogen previously reported for gray matter may, therefore, reflect a greater degree of contamination by white matter and myelin than that achieved in this investigation. The age of the brains from which the samples were taken is also a factor in evaluating the low values obtained since it has been demonstrated that, after 30 yr of age, the amount of ethanolamine plasmalogen begins to decrease with age in human brain (26). The samples used in this investigation were taken from brains 55-75 yr old.

No more than trace amounts of choline plasmalogen were detected in the normal brain gray matter samples (Table I) which is in agreement with previously published results (27). Choline plasmalogen is also apparently not present in more than trace amounts in human white matter as well (27).

The results of the compositional analysis of human brain tumor samples are also in Table I. In all the tumors analyzed, sphingomyelin was present at a higher concentration than in normal gray matter. Choline phosphoglyceride

was increased in most of the tumors compared to the control, whereas the diacyl component of the ethanolamine phosphoglyceride of the tumors was 50-65% lower than that of normal human brain. The amounts of inositol and serine phosphoglycerides in the tumors were not significantly different from those of the controls.

The amount of ether-linked phospholipids found in ethanolamine and choline phosphoglyceride fractions of the tumors was generally higher than that found in normal brain (Table I). Particularly striking were the substantial levels of choline plasmalogen found in virtually all of the brain tumors. This phospholipid was present in only trace amounts in gray matter. The proportions of alkylacyl glycerophosphorylcholine and alkylacyl glycerophosphorylethanolamine were also increased in most of the tumors. The one notable exception was the acoustic neurinoma, which contained only low levels of both alkyl and alkenyl phospholipids. All other tumor groups had on the average at least a 45% increase in total ether-linked phospholipid content.

These results indicate that human brain tumors of the five classes analyzed in this investigation differ considerably from normal human brain gray matter in their phospholipid composition. It is well documented (7,28,29) that human brain tumors contain relatively large amounts of choline-containing phosphoglycerides and correspondingly low levels of ethanolamine phosphoglycerides. The results reported here are in agreement with those findings and extend them by demonstrating that the decrease in ethanolamine phosphoglycerides is due to a decrease only in the diacyl component and not to a decrease in the ether-linked ethanolamine phosphoglycerides. Furthermore, the increase in the choline phosphoglycerides of the tumors is due in part to an increase in the amount of ether-linked choline phosphoglycerides present as well as to an increase in the diacyl component. The net results of these alterations is, therefore, an increase in the proportion of ether-linked phospholipids in human brain tumors.

Analysis of the glyceride fraction of a number of the tumors revealed that of the tumors analyzed all had substantial levels of ether-linked lipid present. The neutral glyceride fraction which amounted to between 12.4 and 17.4% of the total moles of phospho- and neutral glycerol lipids of eleven glioblastomas, four astrocytomas, and one oligodendroglioma contained an average of 1.6 (±0.4), 2.9 (±1.2), and 4.8%, respectively, of the total moles of neutral glycerides as alkyl diacyl glycerol. The same fractions also contained 3.8 (±0.8), 10.1 (±6.9), and 7.4% alkenyl diacyl glycerol, respectively. The one analyzed sample of neutral lipid from normal human brain (7.2% of the total phospho- and triradylglycerol lipids) contained only 0.8% of its total neutral glycerides as alkyl diacyl glycerol and 1.5% as alkenyl diacyl glycerol. This is in general agreement with the results reported by Snyder and Wood from their analysis of normal human brain neutral lipids (1). Comparison of the normal and tumor values indicate that, as with the phospholipid fraction, the neutral glyceride fraction of the tumors contain higher amounts of ether-linked lipids than does the corresponding fraction from normal brain.

A complicating factor inherent in the type of analysis reported here is the wide variety of tumor classes encountered. In addition to the different tumor types presumably reflecting the cell type origin, the tumors were at various stages of malignancy. The astrocytomas analyzed were classified as moderately malignant whereas the glioblastomas ranged from moderately to highly malignant. These factors probably account for the wide variance found between tumors in most of the analyses. In spite of these variations, these results indicate that most tumors have increased levels of ether-linked lipids, particularly choline plasmalogen. Although it is not clear what direct effect increased concentrations of ether-linked lipids may have on neoplastic cells, it does seem probable that the increases in concentration reflect alterations in control mechanisms of lipid metabolism. Thus, the presence in tumors of considerable amounts of choline plasmalogen which is present in only trace amounts in normal brain may reflect a broadening of the specificity of ether-linked phospholipid biosynthesis and a lack of its control in tumor cells. Future investigations of the apparent defective control of ether-linked lipid biosynthesis in brain tumor cells may, therefore, provide some understanding of the overall breakdown of metabolic control which is a general characteristic of neoplastic development (30).

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The Effect of Lipids on Taurocholate Absorption from Intestinal Loops in the Rat

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ABSTRACT

The rates of uptake and serosal transfer of [^{14}C]-labelled taurocholate (7.77 mM in bicarbonate buffer, pH 6.5) were determined in situ in ligated segments of rat intestine in the presence of lipids. Oleic acid, monoolein, lecithin, and lysolecithin enhanced taurocholate uptake and transfer in the jejunum, each lipid exhibiting an optimal concentration at which the bile acid fluxes were maximal. The maximal rates of bile acid uptake observed with the various lipids were close to four times the uptake rates found with the lipid-free taurocholate medium, whereas serosal transfer rates under optimal conditions were enhanced about six-fold. The optimal concentrations differed widely among the various lipids, being inversely related to the lipids' polarity. Simultaneous measurement of taurocholate and [^{3}H]-labelled oleic acid showed that under optimal conditions, when the molar concentration of oleic acid was about equal to that of the bile acid, the fatty acid and bile acid also exhibited closely similar rates of absorption. At other fatty acid concentrations, the fractional rate of absorption of the bile acid was much lower than that of the fatty acid. The rates of uptake and serosal transfer of pure taurocholate by the ileum exceeded those of the jejunum by factors of about 7 and 15, respectively, but in the presence of lipids this difference in absorptive capacity for bile acid between the distal and proximal segment largely disappeared.

INTRODUCTION

Bile acids are absorbed by passive diffusion at all levels of the intestine, while active transport has been reported only in the ileum (1,2). The latter segment has been considered to represent the main site of absorption in the intact animal, but recent reports in humans (3), rats (4), and chickens (5,6) have indicated that the jejunum plays an important role in overall bile acid absorption.

The role of lipids in the uptake of bile acids is not clear. Lecithin has been reported to decrease the jejunal uptake of taurocholate (TC) in vivo (2) and that of taurodeoxycholate in vitro (7).

This report describes the mucosal uptake and serosal transfer of TC by ligated segments of rat intestine in the presence of various lipids at different concentrations.

METHODS

Chemicals

Tauro(carbonyl-^{14}C)cholic acid (51.2 mCi/mmol) and (9,10-^{3}H)oleic acid (2000 mCi/mmol) were from the Radiochemical Centre, Amersham. Taurocholic acid, oleic acid, monoolein (ca. 90% 1-isomer), lecithin from egg yolk, and lysolecithin from egg yolk lecithin were obtained from the Sigma Chemical Co. (St. Louis, MO).

Preparation of the Incubation Media

Weighed amounts of lipids were dissolved in chloroform in a small vial, labelled compounds were added in chloroform or ethanol, and the solvents were removed with nitrogen. Bicarbonate buffer (0.176 mM) containing 7.77 mM TC was added to give the required lipid concentration, the solution was gassed with 5% CO_2 in O_2 and held at 35-7 C overnight. The pH was then adjusted to 6.5 and the solution regassed as before until use.

Measurement of Tissue Uptake

Male rats of a Charles River-derived strain, weighing 150-300 g, were anesthetized with sodium pentobarbital. The upper jejunum, and in some cases the ileum, were exposed and ligated as previously described (4). The ligated segments with their intact vascular and nervous systems were flushed with 0.9% NaCl at 37 C, and injected with the incubation mixture (which contained 0.05 μCi ^{14}C-TC or 0.1 μCi ^{3}H-OA). The incubation period was 15 min. At the end of each incubation, the exact position and length of each loop were determined. The jejunal loops used in the experiments were 10 ± 2 cm long (mean ± SD) and the proximal end was 10 ± 3 cm from the pylorus. The length of the ileal segments was 11 ± 1 cm and the distal end was at a distance of 4 ± 1 cm from the cecal junction. At the end of the incubation period, the loops were removed and

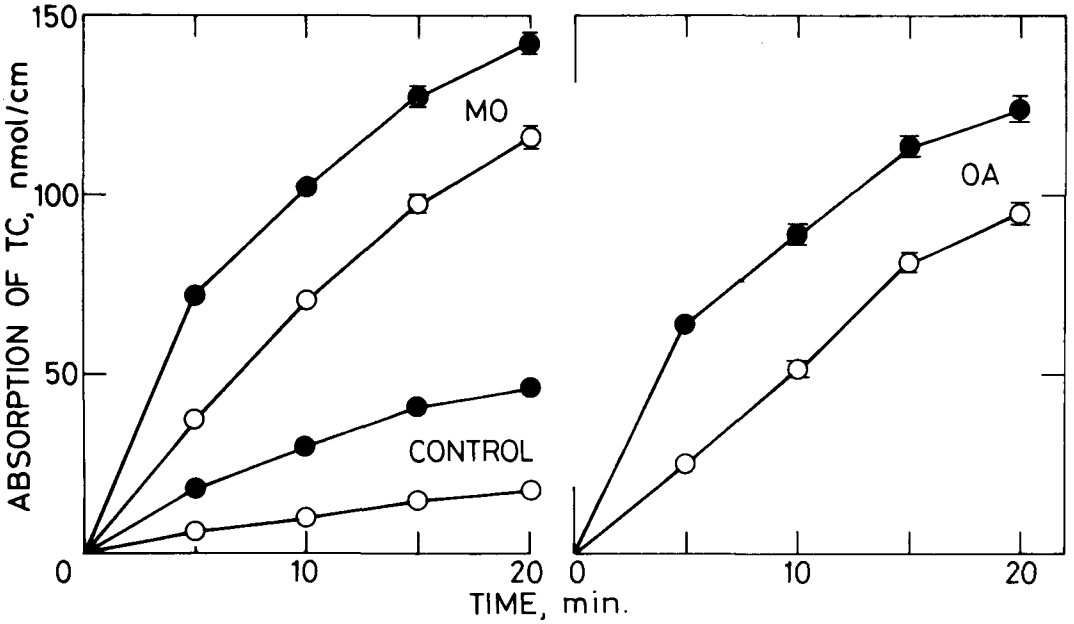

FIG. 1. Time course of absorption of taurocholic acid (TC) by rat jejunal loops without lipids and with added monoolein (MO, 0.5 mM) or oleic acid (OA, 3.9 mM). Experimental conditions were as described in Methods. Black circles represent mucosal uptake, empty circles serosal transfer. Values are means of three rats each; SEM are shown where they do not fall within the circles.

their contents were collected by repeated rinsing with cold 0.9% NaCl. The remaining tissue was homogenized in a high speed homogenizer (Janke & Kunkel, KG, Stauffen i. Br.). Portions of the intestinal contents and tissue homogenate were lyophilised and digested with Soluene (Packard Instr. Inc., Downers Grove, IL). The amount of label was determined by liquid scintillation, using a scintillation mixture containing (g/l): 4 PPO, 0.15 dimethyl POPOP, 330 Triton-X100, and toluene to 1 l. Where the two isotopes were determined simultaneously, two appropriate channels were used, and the contribution of each isotope to the channel of the other was determined using internal standards. Quench correction was calculated in all cases using internal standards of both isotopes.

Calculations

Mucosal uptake was defined as the amount of labelled substrate disappearing from the lumen per cm of segment and was expressed in nmoles/cm/min. Serosal transfer was taken to be the difference between the amount of label disappearing from the incubation medium and the amount of label remaining in the intestinal tissue and was likewise expressed in nmoles/cm/min. The amount of label recovered from the lumen immediately after injection of the medium was 98.3 ± 0.2% (mean ± SD) of the injected dose, and the radioactivity found in the lumen after incubation was corrected for this recovery value.

RESULTS

The mucosal uptake and serosal transfer of TC were determined as a function of incubation time, with and without added oleic acid (OA) or monolein (MO) (Fig. 1). Total uptake and transfer increased over the 20-min period of incubation, with a noticeable decline in the flux rates after 15 min. Transport rates were considerably greater in the presence of lipids at the concentrations tested, as compared to the rates found with the lipid-free medium.

The effect of lipids on the transport rates of TC depended on the nature of the lipids and their concentration (Fig. 2). With oleic acid, TC uptake and transfer increased rapidly as the concentration of the fatty acid rose above 1.8 mM. Maximum flux rates were observed at 7.09 mM OA, and further addition of OA lowered the rates of uptake and transfer of TC. MO, phosphatidylcholine (PC), and lysophosphatidylcholine (LY) produced effects essentially similar to those found with OA, except that the maximal rates of TC transport were achieved at lower concentrations of lipids: 1.4, 0.23, and 0.09 mM for MO, PC, and LY,

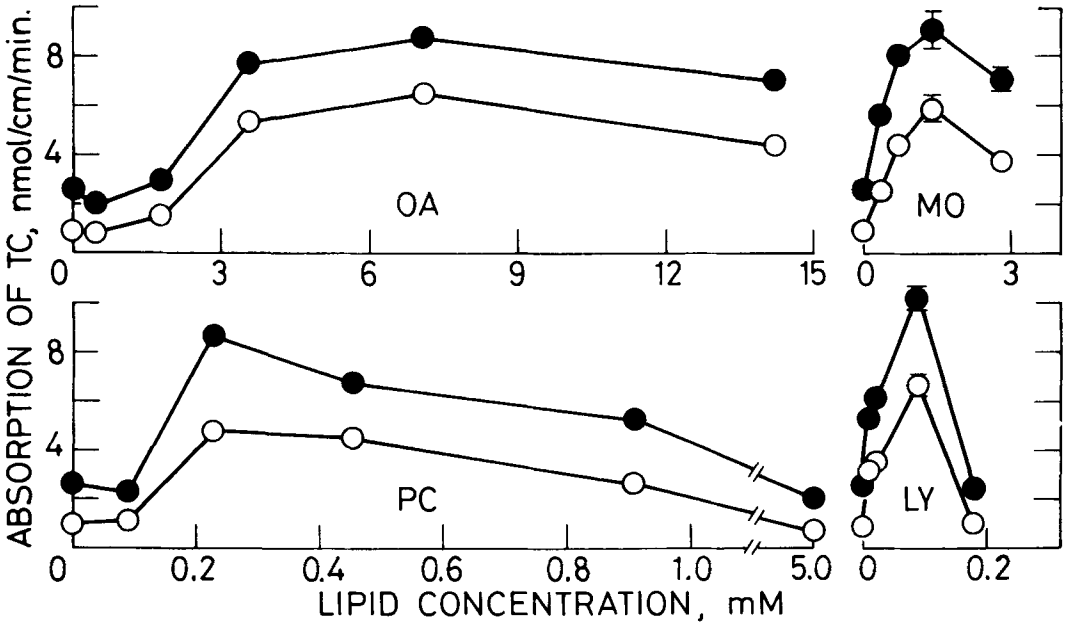

FIG. 2. Absorption of taurocholic acid (TC) by rat jejunal loops in the presence of varying concentrations of oleic acid (OA), monoolein (MO), lecithin (PC), and lysolecithin (LY). Black circles represent mucosal uptake, empty circles serosal transfer. Values obtained in the absence of lipids are means of twelve rats, other values are means of three to four rats each. SEM are shown where they do not fall within the circles. Experimental conditions were as described in Methods. Note difference in lipid concentration scales between top and bottom scales.

TABLE I

Fractional Mucosal Uptake and Serosal Transfer (nmol/mmol/cm/min) of Taurocholic Acid (TC) and Oleic Acid (OA) from Rat Jejunal Loops in situ[a]

Concentration of OA, mM	Fractional mucosal uptake		Fractional serosal transfer	
	TC	OA	TC	OA
1.77	0.76 ± 0.08[b]	1.96 ± 0.20	0.41 ± 0.05	1.58 ± 0.25
7.09	2.50 ± 0.12	2.93 ± 0.16	1.66 ± 0.08	1.60 ± 0.07
14.2	1.81 ± 0.08	2.74 ± 0.51	1.15 ± 0.07	1.44 ± 0.13

[a]The basal medium introduced into the loops contained 7.77 mM TC and 0.05 μCi tauro(carbonyl-^{14}C) cholic acid in 0.176 mM sodium bicarbonate buffer, pH 6.5, in a total volume of 0.5 ml. Oleic acid, added at the concentrations shown, was labelled with 0.1 μCi (9,10-^{3}H)oleic acid. Incubation was for 15 min.

[b]Results are means ± 1 SEM of three rats.

respectively. PC was also tested at a concentration of 5 mM, well beyond the range where PC transport was maximal. Under these conditions, PC uptake and transfer were depressed by 25 and 30%, respectively, below the rates found with the lipid-free medium.

The maximal rates of TC uptake observed in the presence of the different lipids were remarkably constant (3.4-3.9 times the TC uptake from the lipid-free medium). Maximal serosal transfer rates were 5.2-7.4 times the rates found in the absence of lipids.

An additional series of in situ incubations was undertaken in which varying amounts of OA and a tracer dose of ^{3}H-oleic acid were included in the medium. This made possible the measurement of uptake and serosal transfer of both TC and OA. Since the concentration of OA varied while that of TC remained constant, fractional uptake and transfer rates were calculated for purposes of comparison. The results, shown in Table I, confirm the greatly increased rates of TC uptake and transfer at 7.09 mM OA. At that same OA concentration, the fractional uptake and transfer of OA were also highest, although the fractional absorption of the fatty acid was less affected by concentration changes, compared to TC.

TABLE II

Mucosal Uptake and Serosal Transfer of Taurocholic Acid (TC) from Rat Jejunal and Ileal Loops in situ[a]

Lipid added	N[b]	Jejunum		Ileum	
		Mucosal uptake	Serosal transfer	Mucosal uptake	Serosal transfer
		nmol/cm/min			
None	8	2.61 ± 0.14	0.97 ± 0.11	18.1 ± 1.2	15.0 ± 1.1
3.54 mM OA	8	8.04 ± 0.31	4.44 ± 0.38	14.6 ± 1.9	12.5 ± 1.7
0.70 mM MO	4	9.02 ± 0.74	5.94 ± 0.54	15.6 ± 2.1	14.5 ± 1.8

[a]The basal medium introduced into the loops contained 7.77 mM TC and 0.05 μCi tauro(carbonyl-^{14}C) cholic acid in 0.176 mM sodium bicarbonate buffer, pH 6.5, in a total volume of 0.5 ml. Incubation time was 15 min. Results are means ± 1 SEM.

[b]Number of rats tested.

Attempts to increase the TC transport rates beyond the maxima shown in Figure 1 and Table I, by adding mixtures of lipids to the medium, were unsuccessful. For instance, the addition of 3.54 mM OA to a medium containing 1.4 mM MO, the optimal MO concentration for TC transport, did not increase the TC uptake beyond that found with 1.4 mM MO alone, while serosal transport was lowered one-third. A mixture of 0.35 mM MO and 1.77 mM OA slightly increased TC transport beyond the values obtained with each lipid separately, and further addition of 0.45 mM lecithin caused a slight additional increase in TC transport, but none of these values reached the range of maximal rates recorded in Figure 1 for TC transport with different lipids.

TC fluxes were much greater in the ileum than in the jejunum (Table II). The difference was particularly striking in the absence of lipids, when the rate of TC uptake in the ileum was seven times that in the jejunum, and serosal transfer was fifteen times faster in the ileal loop than in the jejunal segment. The addition of OA or MO slightly depressed ileal transport rates while strongly increasing TC fluxes in the jejunum. As a result, in the presence of lipids, the jejunal TC uptake was about 60% of the ileal uptake, and the serosal transfer in the jejunum was 35-41% of that in the ileum.

DISCUSSION

The results reported here show that the rate of transport of TC across the jejunal wall is dependent on the type and concentration of lipid added to the luminal medium, and that for each lipid there is a characteristic "optimal" concentration for maximal TC transport. Lipids in large excess of optimal concentrations may depress TC absorption even below the values observed with the lipid-free buffered TC solution, as in the case of lecithin in Figure 2. A similar situation appears to have prevailed in the experiments by Schiff et al. (2) and Wilson and Dietschy (7), who concluded that the added lipid reduced the absorption of bile acids in the rat jejunum.

An inverse relation was noted between the optimal lipid concentrations for TC uptake and the polarity of the lipids. Since the ability to expand bile acid micelles varies directly with the polarity of the lipids (8,9), the above-mentioned lipid concentration effect may reflect the existence of an optimal micelle size for TC absorption. Intrapolation of Small's data on micelle size (9) yields a molecular micelle weight of roughly 6000 for MO/TC micelles at the optimal MO concentration, and an optimal micellar size of the same order is obtained for lecithin/TC micelles. Small's measurements on OA, carried out at pH 10, yield an estimate of roughly 10,000 for the molecular micellar weight of OA/TC micelles under optimal conditions, but the ionized fatty acid would presumably cause greater micelle expansion than the protonated species prevailing at pH 6.5. Thus, the mixed micelles formed by TC with the above lipids at their optimal concentrations would be expected to be relatively uniform in size, roughly of the order of three times the weight of pure TC micelles, listed as 2400 (9). The narrow range of maximal rates of TC uptake found with different lipids, i.e., 8.7-10.2 nmol/cm/min (see Fig. 2), is consistent with the concept of an optimal micelle size for TC transport.

An additional feature of the lipid-TC micelle under conditions of maximal TC uptake was revealed by measuring the simultaneous absorption of TC and OA (Table I). Rates of uptake and transfer differed widely for the two compounds, except when the fatty acid concentration approached that of the bile acid and the rate of absorption of TC became maximal. Under these conditions, the rate of absorption

of OA was also maximal and close to the TC absorption rate. It is tempting to conclude from these results that at the optimal ratio of OA and TC, micelles are formed which are taken up intact by the mucosal cell. However, according to recent evidence (7,10), the limiting process in the absorption of micelle components is the rate of passage of the micelle through the unstirred water layer adjacent to the brush border, and actual uptake is in the form of monomers after breakdown of the micelle. Whatever the exact mechanism by which lipids and bile acids are transported across the jejunal mucosa, it appears from the present results that the size of the lipid-bile acid micelles plays an important part in the process.

Luminal lipid concentrations were not determined in the present study, but data from a previous publication (4) indicate that the concentrations of free fatty acids and monoglycerides in the undiluted aqueous phase of the upper jejunal contents are about 6.6 and 1.6 mM, respectively. These values are very close to the optimal concentrations of OA and MO found in the present experiments. We suggest, therefore, that in spite of the greater capacity of the ileum for TC absorption (Table II), bile acids are reabsorbed largely in the proximal half of the small intestine in hens (5), chicks (6), and rats (4) because of the pronounced enhancement of absorption caused by lipid digestion products in the jejunum, but not in the ileum. It is clear that this hypothesis requires further testing under a broader range of conditions, with different concentrations of conjugated and unconjugated bile acid species, and taking into account the more alkaline pH prevailing in the ileum.

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Tissue Variation in Hydrocarbon Composition in the Rabbit[1]

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ABSTRACT

This study, which deals with the distribution of hydrocarbons in seven types of rabbit tissues, was done for the purpose of providing information that might help shed light on the biological relevance of the hydrocarbons in mammalian metabolism. Liver, kidneys, brain, spleen, skeletal muscle, perinephric adipose, and a sample of blood serum were collected from a single animal for analysis of their hydrocarbon composition. The analytical methodology consisted of solvent extraction, saponification (adipose), elution chromatography on hydrated alumina, and combined gas chromatography-mass spectrometry. Hydrocarbons were detected in all of the tissues examined at concentrations estimated to range from 0.1 to 0.01% of the total lipid extracted. Three quite distinct distribution modes were recognized. The bulk of the identified components consisted of normal, saturated, nonterpenoid hydrocarbons in the C_{16} to C_{33} range. Squalene, phytene, phytadiene, and pristane were the only terpenoids detected. Nonterpenoid branched (*iso* and *anteiso*) hydrocarbons were identified unequivocally and in significant amounts in the muscle only. The adipose was the only tissue which was relatively rich in monoalkenes, and its overall hydrocarbon composition closely resembled that of the feed. The results of the study are not consistent with metabolic inertness. The observed qualitative and quantitative differences might reflect function and metabolic activities of the individual organs in a way yet to be elucidated.

INTRODUCTION

In 1906 Tsujimoto isolated for the first time from an animal tissue a hydrocarbon which 10 yr later was identified as squalene (1). Since then hydrocarbons have been found in several higher animals and humans (2-8). It is now clear that this chemical species is ubiquitous throughout the animal kingdom. Virtually all possible hydrocarbon structures have been detected in lipid extracts of animal tissues. Such structures include normal, branched (*iso* and *anteiso*), and cyclic alkanes; alkenes; and terpenoid hydrocarbons.

Whereas origin and functions of the hydrocarbons in the vegetable kingdom have been reasonably well elaborated and the relative biosynthetic pathways have been developed (9-12), origin and functions of the hydrocarbons in animal organisms are still largely obscure. We do not know whether they are normal products of metabolism or whether they form after death of the animal; in either case, their precursors are unknown. Even the fundamental question of endogenous vs. exogenous origin has not yet been answered.

Saturated hydrocarbons have been associated with a human pathological condition called splenic follicular lipidosis, characterized by abnormal accumulation of paraffinic hydrocarbons in the spleen up to 16.3% of the total lipids (5). Gazzarrini and Nagy (13) and Brooks et al. (14) identified *n*-alkanes in human atherosclerotic plaques. Various types of hydrocarbons were detected in the fat globule membrane of human and bovine milk (15), in rat liver cell membranes (3), and in all human serum lipoprotein classes, with the highest relative amount (4.4% of the total lipid) in the ultracentrifugal residue (16). Cain et al. (17) found significant quantitative differences between the hydrocarbon composition of human brain meninges and that of the analogous neoplastic tissue (meningiomas). All of these observations suggest that the hydrocarbons may have an active metabolic role in mammals, or even a structural function in biological membranes. Other data from the literature also suggest that the various classes of hydrocarbons might not be uniformly distributed among all the anatomical sites of a single animal. Thus, Bandurski and Nagy (8) determined that the hydrocarbons in beef heart muscle consisted mainly of C_{14} to C_{35} *n*-alkanes, whereas Nagy et al. (7) showed that in bovine liver they consisted primarily of branched/cyclic alkanes and only trace quantities of *n*-alkanes. Finally, Nicholas and Bombaugh (18) reported that beef brain contains predominantly branched alkanes. However, no preferential distribution had been demonstrated within a single animal. Consequently, it was not

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known whether this apparent selectivity might have biological significance. The study of the distribution of hydrocarbons in different anatomical sites of a single animal should help clarify this point, and might be a first step toward solving the problem of the origin and role of hydrocarbons in mammalian metabolism. In view of a possible biochemical relationship between hydrocarbons and fatty acids and of the interest in this laboratory in fatty acid metabolism, a comparative study of hydrocarbon distribution in seven types of rabbit tissues was conducted. Liver, kidneys, brain, spleen, muscle, adipose, and a sample of blood serum were collected from a single animal and analyzed for hydrocarbon composition by using elution chromatography and combined gas chromatography-mass spectrometry (GS-MS). The results of this investigation show that there are indeed marked differences in the qualitative and quantitative hydrocarbon composition among the various organs. Whether or not such differences are biologically relevant can be decided only upon further experimentation.

EXPERIMENTAL PROCEDURES

Great caution was exercised throughout the experiments to avoid contamination. All glassware was cleaned with hot concentrated $H_2SO_4/K_2Cr_2O_7$ or warm freshly prepared 15% methanolic KOH. No stopcock grease was used in the laboratory, and the samples were not allowed to come in contact with plastic or rubber. Scalpels, scissors, and blades were thoroughly rinsed with chloroform before use. All solvents were Fisher certified (Fisher Scientific Co., Silver Spring, MD) and freshly distilled. Reagent grade acid aluminum oxide powder (J.T. Baker Chemical Co., Phillipsburg, NJ) was used for elution chromatography after hydration to 6% water content. Vitamin A used for the preparation of anhydrovitamin A was purchased from Research Plus Laboratories, Inc., Denville, NJ.

The subject was an adult male rabbit which was maintained on Purina Rabbit Chow (Ralston Purina Co., St. Louis, MO) and sacrificed with excess anesthetic. Blood was obtained by heart puncture under Nembutal anesthesia. Kidneys (16.5 g), brain (7.5 g), and spleen (1.4 g) were used in their entirety. The adipose tissue (24 g) was all obtained from the perinephric cavity, and the muscle (50 g) was obtained from the hind thighs. Approximately half of the liver (40 g) was used. The organs were rapidly excised after sacrifice; they were washed with water, blotted dry, wrapped in solvent-washed aluminum foil, and stored at -40 C until used.

Extraction and Isolation of the Hydrocarbon Fraction

The tissues were first cut into small pieces, then homogenized twice in a Sorvall omni-mixer at 0 C for 4-min periods with a total of 30 volumes of chloroform-methanol 2:1. The lipid extracted was transferred into hexane by a modification of a previously published procedure (19). Namely, the chloroform-methanol extract was evaporated to one-fifth of its original volume under reduced pressure in a rotavapor; the concentrate was added to about 200 ml of hexane, and the volume reduced to about 20 ml under reduced pressure. This dilution-evaporation process was repeated three or four times with extra addition of 10 to 20 ml of benzene each time to speed up the elimination of moisture. The resulting hexane solution was washed five times with a 0.5% aqueous solution of Na_2SO_4 and once with water in a separatory funnel, and finally dried over anhydrous Na_2SO_4. The amount of crude lipid in the extract was determined by weighing the residue in an aliquot after solvent had evaporated. The hydrocarbon fraction was obtained by column chromatography on hydrated alumina of the residue after solvent evaporation. The eluent was hexane, and the ratio alumina to sample was about 100:1. The hydrocarbons were completely eluted within the first 5 to 6 column volumes, and all of the fractions containing hydrocarbons were combined and analyzed by GC-MS. In all cases, the alumina columns were extensively irrigated with hexane prior to deposition of the crude sample at the top. The washings, when checked by GC-MS, were invariable found to be free from contaminating hydrocarbons.

Prior to column chromatography, the crude extract of the adipose tissue was evaporated under a stream of nitrogen at 30 C to a thick oil. This was saponified without a solvent according to the procedure of Schwartz et al. (20). After being cooled to room temperature, the soap was ground in a mortar into a fine powder which was extracted four times with hexane in centrifuge tubes. The supernatant fractions were decanted, combined, and then dried over anhydrous Na_2SO_4. The residue obtained after solvent evaporation was chromatographed on hydrated alumina as described above for the other extracts.

Freshly drawn whole blood was allowed to coagulate by storage at 0 C for 2 hr. Serum was separated by centrifugation at 1260 *g* for 30 min at 10 C. The serum lipids were extracted with chloroform-methanol according to a procedure described by Nelson (21). The

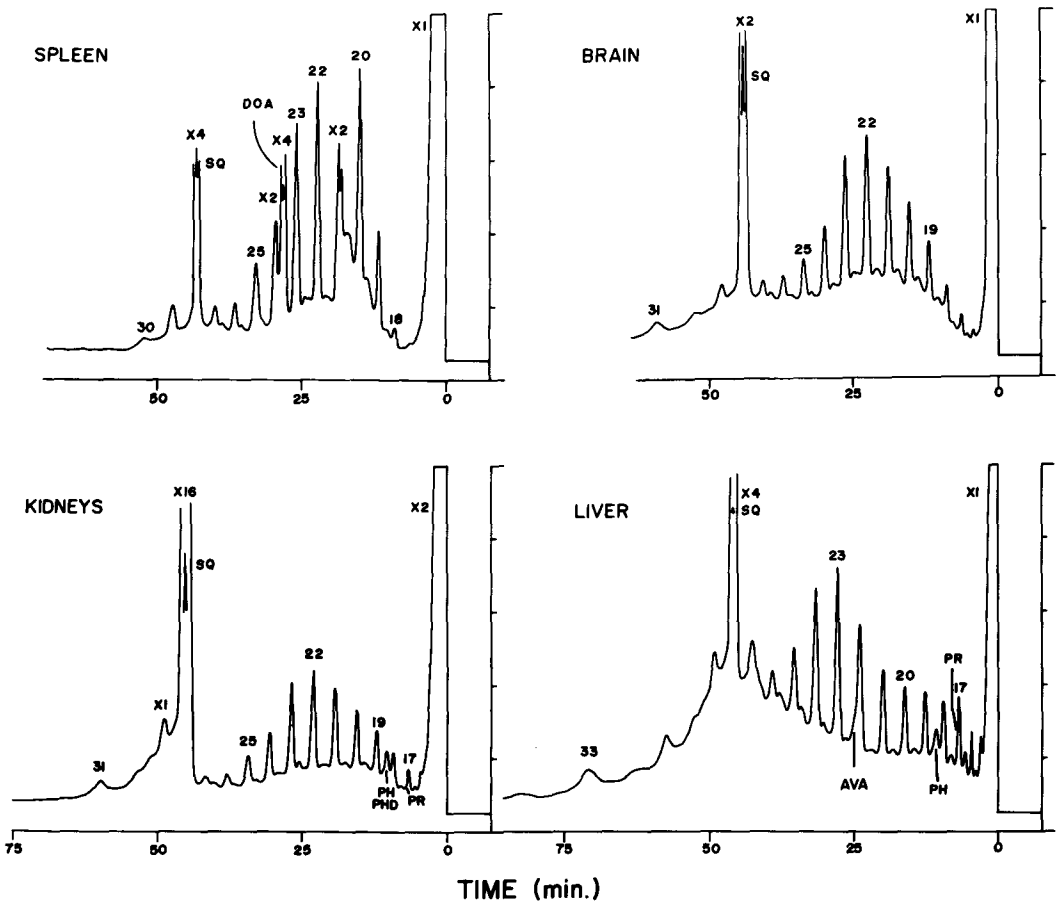

FIG. 1. Gas liquid chromatograms of the hydrocarbon fraction of four rabbit tissues. SQ = squalene; PH = phytene; PHD = phytadiene; PR = pristane; AVA = anhydrovitamin A; DOA = dioctyl adipate. For conditions, see Experimental Procedures.

crude extract obtained after solvent evaporation was subjected to elution chromatography as usual.

Ten g of Purina Rabbit Chow was finely ground in a mortar, suspended in 60 ml of methanol in a Sorvall omni-mixer and homogenized for 1 min. Then 120 ml of chloroform was added and homogenization was continued for 3 more min. After filtration, the solid material was homogenized once again with 150 ml of chloroform-methanol 2:1 for 4 min. The combined filtrates were shaken in a separatory funnel with 75 ml of 0.88% KCl, then with 75 ml of water. The lower (organic) phase was clarified by being passed through a plug of anhydrous sodium sulfate. The residue, after solvent evaporation, was completely soluble in hexane, and the hydrocarbon fraction was isolated as usual. From 10 g of chow, we obtained 0.4842 g of total fat, which yielded 7.0 mg of hydrocarbon fraction.

Analysis by Gas Liquid Chromatography-Mass Spectrometry (GLC-MS)

The GLC analysis was done by using a Varian Aerograph model 1520 gas chromatograph equipped with a flame ionization detector. The column was stainless steel, 1.05 m in length x 3.2 mm O.D., treated with dimethylchlorosilane, and packed with 3% JXR on 80-100 mesh Gas Chrom Q (Applied Science Laboratories, State College, PA). The temperature was programmed from 125 to 225 C at a rate of 2 C/min. The carrier gas (He) was supplied at a head pressure of 2.8 kg/cm^2 and the flow rate was about 20 ml/min. The GLC column was connected by a heated (200 C) glass-lined stainless steel transfer line, 1 ft long, and a Watson-Biemann separator to a Hitachi RMU-6E single focusing mass spectrometer, operating at an ionizing energy of 70 eV, source temperature 250 C, accelerating voltage 1.8 kv, separator temperature 200 C. The sample was

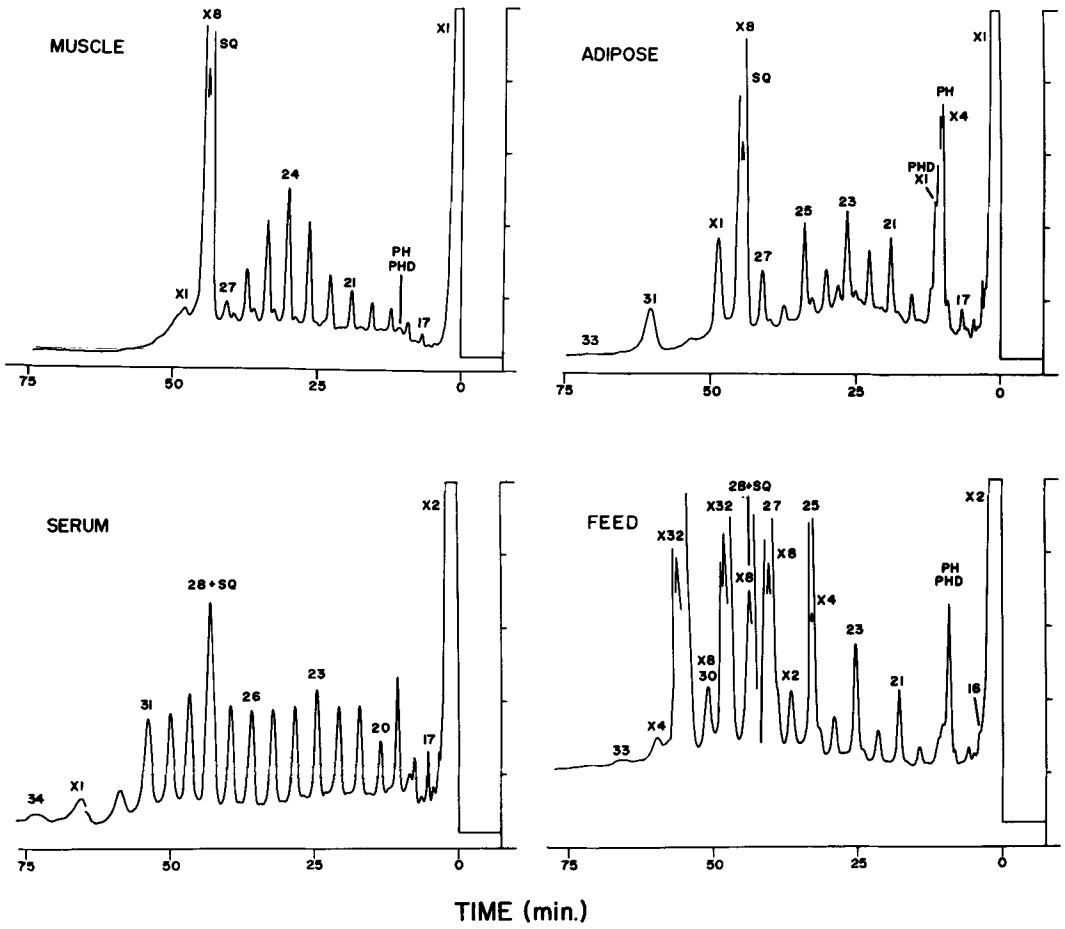

FIG. 2. Gas liquid chromatograms of the hydrocarbon fraction of three rabbit tissues and feed (Purina Rabbit Chow). SQ = squalene; PH = phytene; PHD = phytadiene. For conditions, see Experimental Procedures.

introduced into the gas chromatograph in hexane solution; half of the effluent went to the flame detector and half was introduced into the mass spectrometer via the separator. The mass scans were made in 7 sec.

Identification of unknowns was done exclusively on the basis of mass spectrometry. A hydrocarbon was assumed to be present only when the relative molecular ion was clearly recognizable along with the appropriate fragmentation pattern. The mass spectra of all the terpenoids except that of anhydrovitamin A were compared with spectra published in the literature. Anhydrovitamin A was prepared by acid-catalyzed dehydration of vitamin A (22), and the mass spectrum of the purified material (alumina) was compared with that of the unknown.

Solvent Controls

The following controls were run in order to ascertain that none of the solvents used was contaminated with hydrocarbons. Five hundred ml of hexane and 200 ml of chloroform-methanol 2:1 were evaporated to 10 μl and the residues were gas chromatographed. Five hundred ml of deionized water was shaken twice with chloroform in a separatory funnel. The chloroform phase was evaporated as usual and the residue was chromatographed. All the residues were found to be completely free from hydrocarbons.

RESULTS

The quantities of each tissue/organ indicated above afforded the following amounts of total lipid extracts (expressed in grams): kidneys 0.626; brain 0.556; spleen 0.038; adipose 21.0; muscle 0.700; liver 1.760. From 20 ml of blood serum, we obtained 40 mg of total lipid. The hydrocarbon concentration in all tissues

examined was estimated to range from 0.1 to 0.01% of the total lipid extracted. Figures 1 and 2 illustrate the distribution of hydrocarbons in the tissues analyzed. All the prominent peaks were positively identified as straight-chain alkanes. Some of the peaks in Figures 1 and 2 were labeled to aid in the identification of the eluted compounds. The C_{28} *n*-alkane eluted together with squalene under the experimental conditions used. Not enough material was available to allow separation of branched-chain and *n*-alkanes prior to GLC-MS. It is clear that, if one excludes squalene, the overwhelming majority of the hydrocarbons consisted of *n*-alkanes. The baseline hump, which is particularly pronounced in the chromatograms of brain and liver extracts, and which is almost invariably observed in chromatograms of naturally occurring hydrocarbon fractions, may be due to the presence of small quantities of several unresolved branched isomers. Specific nonterpenoid branched (*iso* and *anteiso*) hydrocarbons (C_{24} to C_{27}) could be unequivocally identified in the muscle only, obviously due to their presence there in significant amounts. They were eluted ahead of their *n*-isomers and could be clearly associated with the small but prominent peaks between the major ones (Fig. 2). C_{17} through C_{27} monoalkenes were detected in the adipose tissue; they were eluted less than 1 min ahead of their saturated analogs. The C_{17} and C_{18} monoalkenes were identified in liver and muscle. No alkenes were detected in any of the other tissues. In Purina Rabbit Chow, each *n*-alkane was accompanied by the monounsaturated analog with the exception of the C_{32} and C_{33}. In all cases, it was estimated that each monoalkene amounted to 10% or less of each saturated analog. Pristane (2,6,10,14-tetramethylpentadecane) was detected in liver and kidneys only; it eluted right after the C_{17} *n*-alkane (Fig. 1). In the chromatogram of the liver extract (Fig. 1), the shoulder on the peak corresponding to the C_{22} *n*-alkane was associated with an apparent molecular ion at m/e 268. Its mass spectrum, as it appeared after background subtraction, was indicative of a terpenoid hydrocarbon whose empirical formula would be $C_{20}H_{28}$. This compound was identified as anhydrovitamin A and its identity was confirmed by comparison of its chromatographic behavior and mass spectrum with those of authentic material. The compound associated with the large peak between the C_{23} and C_{24} *n*-alkanes in the chromatogram of the spleen extract (Fig. 1) was dioctyl adipate, the origin of which is unclear.

DISCUSSION

The present study is the first to characterize in detail the hydrocarbon composition of several tissues from a single mammal and shows that there are considerable qualitative and quantitative compositional differences among various anatomical sites. Scrutiny of the chromatograms of Figures 1 and 2 reveals that three distinct distribution patterns are easily recognizable: (a) one where odd-numbered hydrocarbons predominate over the even-numbered ones (adipose); (b) one where the pattern is bell shaped with the apex at C_{22} (kidneys, brain, and spleen), C_{23} (liver), or C_{24} (muscle); (c) one where the distribution of hydrocarbons is quite uniform (serum). One modal distribution only was present in any one tissue. We do not know at this time whether or not tissue hydrocarbon profiles of any organs/tissues in other rabbits are the same as those observed in the animal studied by us. Results of a study conducted at our laboratory using identical methodology have shown that the hydrocarbon compositions of the liver, kidneys, skeletal muscle and adipose from several rats are qualitatively and quantitatively identical for the same type of tissue. We expect that a similar constancy would be observed in the rabbit.

Squalene, the well known biosynthetic precursor of cholesterol, was consistently the most abundant hydrocarbon in all tissues examined except serum. However, if one considers the attenuation factors in all the chromatograms, it is apparent that the brain, along with serum, was the least rich in squalene among all the tissues. Phytene (3,7,11,15-tetramethyl-*n*-hexadec-2-ene) was always accompanied by minor amounts of neophytadiene (3-methylene-7,11,15-trimethyl-*n*-hexadec-1-ene). This terpenoid monoalkene is present in relatively large amount in the adipose tissue where it is the most abundant hydrocarbon after squalene. Its most likely source is the feed where it was also present in fairly large quantity (Fig. 2). The similarity between the overall hydrocarbon distribution in adipose tissue and that in Purina Rabbit Chow is striking not only with respect to the relatively high concentration of phytene which seems to accumulate preferentially in the adipose tissue, but also with regard to the prevalence of odd-numbered over even-numbered hydrocarbons and the presence of monoalkenes. Close chemical similarity between depot fat and dietary fat has been recently demonstrated in the rat (23) with respect to absorption and deposition of *trans* fatty acids.

Owing to the present lack of pertinent experimental data, any discussion on the origin of hydrocarbons in mammalian tissues can only be speculative. In most general terms, the hydrocarbons could be endogenous or exoge-

nous. If they are endogenous, they (a) could arise from direct synthesis (as is the case for squalene) or (b) could be products of normal metabolic processes, which in certain types of derangement, e.g., the splenic follicular lipidosis (5), could result in their accumulation in abnormal quantities in certain tissues. Other pathological conditions (yet unknown) might well bring about changes in the qualitative and quantitative distribution of hydrocarbons in animal tissues. One can even speculate that such changes, if they indeed occur, might have diagnostic value. If the hydrocarbons are entirely synthesized in the tissues, the existence of three different distribution patterns could be interpreted to mean that there are three distinct biosynthetic pathways. If, on the other hand, the hydrocarbons are exogenous, they could originate (a) from the diet or (b) from environmental pollution. The latter hypothesis, in our view, is much less likely than the former, primarily in view of the striking tissue variations observed in the present study.

Hydrocarbons are known to occur in all living organisms, both vegetable and animal. Consequently they must be present in animal diets as well as in human diets. The oxidation of hydrocarbons into fatty acids in animal tissues appears to be a widespread occurrence. Thus, when McCarthy (24) administered [1-^{14}C]octadecane or [1-^{14}C]hexadecane to rats, goats, and chickens, the radioactivity was completely recovered in fatty acids of the same chain lengths as the hydrocarbons administered. Two years later Kolattukudy and Hankin (25) demonstrated the absorption and utilization of dietary *n*-nonacosane ($C_{29}H_{60}$) in the rat. Using tracer techniques, these authors found that the radioactivity became widely distributed in all the tissues and organs examined, and especially the liver. Although ^{14}C was found in all lipid classes in the liver, the bulk of the radioactivity was in the fatty acid portion of the phospholipids. Even if it has been proven that dietary paraffins are absorbed, transferred to various organs, and metabolized into fatty acids in higher animals, there are not yet enough data to establish whether and to what extent the diet contributes to the hydrocarbon deposits in these same animals. Furthermore, the inverse metabolic relationship consisting of the conversion of fatty acids into hydrocarbons has not yet been proven in animal organisms, although the prevalence of odd-numbered alkanes over even-numbered ones in certain tissues prompted some authors to suggest that *n*-alkanes might derive from fatty acid by decarboxylation (7).

Definitive conclusions as to the origin and function of the hydrocarbons in animal tissues must await further experimentation. All we can say at present is that the existence of diverse distribution patterns within an individual animal, as shown in the present study, is not consistent with metabolic inertness. Rather, the observed variations might reflect differences in the metabolic activity of the various tissues in a way yet to be elucidated. We feel that the hypothesis that the hydrocarbons may be involved in lipid metabolism deserves further research effort. Although we cannot draw any definitive conclusions, we hope that the results of the present work will be useful to invite speculation and to stimulate further experimental work.

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Characterization of Bovine Rumen Liquor Isoprenoid Hydrocarbons with Reference to Dietary Phytol

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ABSTRACT

The isoprenoid hydrocarbons, *trans*-phyt-2-ene (82.2%), phyt-1-ene (6.5%), and phytane (11.3%), were detected in bovine rumen liquor by means of $AgNO_3$-impregnated thin layer chromatography and gas liquid chromatography with mass spectrometry and infrared spectrophotometry. The unsaturated hydrocarbons were identified by comparing their chromatographic, physical, and chemical properties with those of synthetic isoprenoid hydrocarbons prepared from dihydrophytol. The natural level of the original isoprenoid components within the rumen is comprised of phytol and dihydrophytol (of the ratio of 2:1). These represented at least 60% of the total free fatty alcohol content of the rumen liquor lipid extract and could, therefore, be the origin of the isoprenoid hydrocarbons observed.

INTRODUCTION

Isoprenoid compounds are widely distributed in nature. In the rumen, these all apparently originate from phytol (*trans*-3,7,11,15-tetramethyl-2-hexadecen-1-ol), an unsaturated isoprenoid alcohol which represents about 30% of the chlorophyll content of dietary green herbage for ruminant animals (1). Phytol can be readily hydrogenated to dihydrophytol (3,7,11,15-tetramethylhexadecan-1-ol) which is oxidized to produce phytanic (3,7,11,15-tetramethylhexadecanoic) acid by the action of microorganisms in rumen liquor (2). Phytanic acid is found in many ruminant tissues (3). More recently, phytol and dihydrophytol were found in milkfat (4) and as dihydrophytyl-phytanate (wax esters) in rumen liquor (5).

Other phytol metabolites, namely phytane (3,7,11,15-tetramethylhexadecane) and isomers of phytene (3,7,11,15-tetramethylhexadecene) hydrocarbons, have been found as minor constituents of milkfat (6) and butterfat (7). This communication reports the occurrence of these isoprenoid hydrocarbons in bovine rumen lipid extracts and discusses their relationship with the dietary phytol source.

MATERIALS AND METHODS

Rumen liquor was obtained from a Jersey cow fitted with a fistula and fed on fresh white clover ad libitum. This animal was fasted overnight before the rumen liquor was taken and strained through cheesecloth and the lipid constituents were extracted with diethyl ether as described before (5). From 100 ml of rumen liquor, a total lipid extract of 312.6 mg was recovered, dissolved in chloroform, and applied to an activated silicic acid column (18 g, Bio-Sil A, Bio-Rad Laboratories, Richmond, CA) for fractionation as outlined earlier (5). This produced the following main fractions of lipid components: fraction 1, hydrocarbons-carotenoids-wax esters-sterol esters (26.4 mg); fraction 2, free fatty acids-sterols-fatty alcohols (179.0 mg) and fraction 3, pigments-phospholipids (92.2 mg).

Isolation of the Hydrocarbons

Hydrocarbons (12.0 mg) were isolated from fraction 1 by preparative thin layer chromatography (TLC) after development with hexane-diethyl ether-acetic acid (85:15:1 v/v).

Isolation of the Free Fatty Alcohols

Fraction 2 was methylated with diazomethane (8) and the resultant FFA-methyl esters were removed by silicic acid (20 g)

TABLE II

Composition of the Hydrocarbon and Free Fatty Alcohol Constituents of Bovine Rumen Liquor

Components	mg/100 ml rumen liquor
Isoprenoid hydrocarbons	
Phytane	0.12
Phyt-1-ene	0.07
cis-Phyt-2-ene	-
trans-Phyt-2-ene	0.88
Other hydrocarbons	
C_{18}-C_{35}	10.93
Total	12.00
Isoprenoid fatty alcohols	
Phytol	12.72
Dihydrophytol	6.36
Other fatty alcohols	
C_{18}-C_{34}	12.72
Total	31.80

column chromatography with chloroform as eluant. Free fatty alcohols (31.8 mg) were isolated from the remaining components of fraction 2 by further fractionation using preparative TLC with benzene as the developing solvent system.

Preparation of Authentic Isoprenoid Hydrocarbons

A range of unsaturated hydrocarbons can be readily obtained by dehydrating the corresponding primary alcohol. In this case, dihydrophytol was prepared by hydrogenating phytol (K and K Laboratories Inc., Plainview, NY) in methanol over PtO_2 at 45 C for 6 hr at atmospheric pressure. Approximately 130 mg dihydrophytol was stirred and heated to 150 C with 0.5 ml 88% *ortho*-phosphoric acid (AR, British Drug Houses Ltd., Poole, England) for 1.5 hr. During this reaction, some charring was noticed so the final material was dissolved in diethyl ether and filtered while transferring it to a separating funnel. The ethereal extract was washed with distilled water until neutral, dried over anhydrous Na_2SO_4, and evaporated to dryness under reduced pressure at 45 C. These dehydration products (120 mg) dissolved in chloroform were applied to an activated silicic acid column (20 g). The isoprenoid hydrocarbons (95.5 mg) were eluted with chloroform (70 ml) and the residual products (20.5 mg) with methanol (100 ml). This reaction produced a satisfactory quantity of unsaturated isoprenoid hydrocarbons that was further resolved by preparative TLC with 10% $AgNO_3$-impregnated purified (9) silica gel G (E. Merck, A.G. Darmstadt, Germany) plates with hexane-diethyl ether (95:5 v/v) as developing solvent system. By this procedure, three different unsaturated isoprenoid hydrocarbon isomers (A, B, and C) were isolated. The proportion of these components together with their chromatographic properties is shown in Table I.

Analysis of Hydrocarbons

The hydrocarbons were analyzed by gas liquid chromatography (GLC) using a dual flame Pye 104 instrument (Pye-Unicam Ltd., Cambridge, England) fitted with standard glass columns (1.5 m x 0.4 cm ID). Two different liquid phases, namely 10% EGSS-X on 60-80 mesh Gas-Chrom Z and 3% JXR on 100-120 mesh Gas-Chrom Q (Applied Science Laboratories, State College, PA) solid supports, were operated at 120 and 160 C with nitrogen carrier gas flow-rates of 20 and 30 ml/min, respectively. To aid the identification of hydrocarbon mixtures, the chromatographic properties of these columns were calibrated with authentic standards and the 3% JXR column coupled

TABLE I

Composition and Chromatographic Properties of Isoprenoid Hydrocarbons Synthesized by the Dehydration of Dihydrophytol

Isoprenoid hydrocarbon	Composition (wt %)	$AgNO_3$-impregnated TLC R_f value[a]	GLC ECL[b] value on 3% JXR at 160 C	GLC ECL[b] value on 10% EGSS-X at 120 C
trans-Phyt-2-ene (A)	15.2	0.95	18.48	18.92
cis-Phyt-2-ene (B)	61.2	0.92	18.35	18.72
Phyt-1-ene (C)	23.6	0.86	17.85	18.18
Phytane	-	0.97	18.14	17.87

[a] R_f value of *n*-$C_{18:0}$ hydrocarbon = 1.00. TLC = thin layer chromatography.
[b] Equivalent chain length values with reference to *n*-hydrocarbons. GLC = gas liquid chromatography.

with a mass spectrometer (GLC-MS) (AEI MS30 Double Beam MS, AEI Scientific Apparatus Ltd., Manchester, England).

Analysis of Fatty Alcohols

The 3% JXR GLC column (as above) was used to analyze the fatty alcohol mixtures either in the free state or as their alcohol-acetate derivatives. Both isothermal conditions (180 C) or temperatures programmed over the 180 to 270 C range at 4 C per min were applied and carrier gas flow rates of 30 ml/min were uniform throughout. For the identification of the fatty alcohol components, their GLC properties were compared with those of authentic standards under identical conditions as outlined elsewhere (5,10).

RESULTS

The three isoprenoid hydrocarbon isomers A, B, and C (Table I) prepared by dehydrating dihydrophytol were initially inspected as films on KBr discs by infrared spectrophotometry (Infracord 137, Perkin-Elmer Ltd., Beaconsfield, Bucks, England). They all revealed standard isoprenoid coupled adsorption bands in the 1360-1370 cm^{-1} region, defined isoprene bands at 737 cm^{-1} and isopropyl bands at 1180 cm^{-1} (7). In particular, the spectrum of compound A also included the presence of a *trans*-double bond (968 cm^{-1}) while compound C differed showing a strong adsorption at 910 cm^{-1} indicating a distinct terminal vinyl group (11).

All these compounds had the same molecular ions at m/e 280 (M) by MS and some specific fragment ions of slightly variable strengths at m/e 252, 210, 196, 181, 140, 125, 111, 97, 70, and 57. Similarly, they all produced phytane [m/e 282 (M) and other fragment ions at m/e 267, 197, 127, and 57 which demonstrated the methyl-branched sites on the molecule (12)] as their only hydrogenation product. The above evidence together with the $AgNO_3$-impregnated TLC and GLC chromatographic properties (Table I) identified compound A as *trans*-phyt-2-ene, B as *cis*-phyt-2-ene and C as phyt-1-ene.

The isoprenoid hydrocarbons present in bovine rumen liquor and identified by GLC-MS showed a distribution pattern per 100 ml rumen liquor of phytane (0.12 mg), phyt-1-ene (0.07 mg), and *trans*-phyt-2-ene (0.88 mg). This represented 8.9% of the total hydrocarbon contents (Table II). The other hydrocarbons, including *n*-nonacosane (C_{29}) and *n*-hentriacontane (C_{31}) as the principal components, resembled those of the white clover neutral lipids (10) as expected.

In contrast, the composition of the fatty alcohol fraction differed remarkably from that of the hydrocarbons in respect to the quantity of isoprenoid components (Table II). Here at least 60% was represented by phytol (40%) and dihydrophytol (20%). The remaining 40% was spread over the C_{18} to C_{32} range with *n*-triacontanol (C_{30}) as the main contributor. This resembled the fatty alcohol distribution pattern of those associated with the neutral lipids of the dietary white clover (10).

DISCUSSION

The microbial action on phytol in rumen liquor has been studied extensively by Patton and Benson (2). Their conclusive observation that dihydrophytol is the biohydrogenation product from the chlorophyll-phytol moiety would also apply with this work and these isoprenoid fatty alcohols are the major contributions to the entire free fatty alcohol contents of the rumen liquor under investigation. No doubt these isoprenoid fatty alcohols (ca. 19.0 mg/100 ml rumen liquor) are also the source of the isoprenoid hydrocarbons (ca. 1.0 mg/100 ml rumen liquor) now found in the rumen.

Phytol can be readily dehydrated catalytically (13) or biologically (14) under mild conditions to yield phytadiene isomers. One of the relevant phytadiene isomers, neophytadiene (3-methylene-7,11,15-trimethylhexadec-1-ene), has been reported by Urbach and Stark (7) to be present as part of the isoprenoid hydrocarbons in both butterfat and ryegrass lipid extracts. They proposed that any dietary ryegrass neophytadiene would be biohydrogenated to form phyt-1-ene as the isoprenoid hydrocarbon constituent of rumen liquor. However, in the present study, the actual findings differed markedly. Initially, the dietary white clover source used provided (10) no isoprenoid hydrocarbon of the same nature as neophytadiene. Secondly, since *trans*-phyt-2-ene was the major isoprenoid hydrocarbon present in rumen liquor, this also suggests that another source of isoprenoid hydrocarbons must be considered.

The ability of the microorganisms within the rumen liquor to produce methane from dietary carbohydrate is well known (15). These conditions would be adequate to allow phytol, with the natural *trans*-2 configuration, to freely exchange its primary hydroxyl-group with hydrogen to form *trans*-phyt-2-ene as a main end-product. Even the proximity of the labile double bond near the terminal hydroxyl-group could also permit the formation of the observed

phyt-1-ene (vinyl-structure) isoprenoid hydrocarbon when hydroxyl-proton interchange takes place.

On the other hand, phyt-1-ene could be alternatively produced as part of the migration process formed during the biohydrogenation action on *trans*-phyt-2-ene to yield phytane as the end-product. Similar isomerization and migration of double bonds during the parallel biohydrogenation of polyunsaturated fatty acids by rumen microorganisms has been established and the distribution pattern of their various products has been recently reinvestigated (16).

The chemical, physical, and chromatographic properties of the unsaturated isoprenoid hydrocarbons are governed by their molecular structure. The steric hindrance imposed on the unsaturated site of the phyt-2-ene isomers reduces the formation of $AgNO_3$-complexes to prevent distinct resolution by TLC between the different structural configurations (i.e., *cis*- or *trans*-isomers) and phytane. However, combinations of $AgNO_3$-impregnated TLC, GLC-MS, and infrared spectrophotometry did resolve the isoprenoid hydrocarbons present in bovine rumen liquor. These results also indicate that the monounsaturated isoprenoid hydrocarbons shown in various ruminant products (6,7) are probably derived from the initial microorganism reaction on dietary phytol and intermediates in the rumen.

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Lipid Metabolism in Plasma, Liver, and Adipose Tissue of Rats with Experimental Chronic Nephrotic Syndrome

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ABSTRACT

Plasma, liver, and adipose tissue lipid composition and synthesis from [1-^{14}C] acetate were studied three months following induction of nephrotic syndrome in rats by injection of antiglomerular basement membrane protein. Plasma triglyceride concentrations and specific radioactivities were elevated, and the triglycerides contained increased proportions of oleic acid. Plasma cholesterol and phospholipid concentrations were also increased, but free fatty acid levels were not. Liver triglyceride concentrations were decreased and incorporation of [1-^{14}] acetate into liver triglycerides was also depressed below that of normal controls. Nephrotic rat liver triglycerides contained a higher proportion of oleic acid and lower arachidonic acid than did controls. Incorporation of [1-^{14}C] acetate into adipose tissue lipids of the nephrotic rats was increased, and the proportion of palmitic acid was decreased. In the chronic nephrotic rat, the major source of the increased plasma triglycerides may be fatty acids mobilized from adipose tissue stores.

INTRODUCTION

Hyperlipemias comparable to those in human nephrotic syndrome have been produced in rats either by injection of purine aminonucleoside (1,2) or antikidney serum (3,4). In both these types of experimental nephrotic syndrome, the lipid abnormalities observed appear to vary with the time elapsed following administration of the inducing agent. In aminonucleoside nephrosis, serum cholesterol, triglycerides, and free fatty acids begin to rise at about the fourth day after initiation of treatment, even though at this time [1-^{14}C] acetyl CoA incorporation into liver and adipose tissue lipids is decreased, the latter due to a probable direct inhibitory effect of the aminonucleoside on synthesis of lipogenic enzymes (2). Peak plasma lipid levels in the latter study were reached after about 17 days, and then began to decline; in another study, however, plasma lipids continued to rise after 22 days, at which time liver synthesis of triglycerides, phospholipids, and cholesterol was found to be above normal (1). In nephrosis induced in rats by antikidney serum, serum lipid concentrations rose to peak values after 4 days and then progressively declined (5). At 5 days, liver cholesterol and total lipid concentrations were found to be normal despite elevations of these lipids in the plasma (6); in another similar study, however, liver triglyceride concentrations were increased at this time point (7). When [1-^{14}C] acetate was injected into rats, and followed 4 hr later by antikidney serum, the specific activities of plasma and liver total fatty acids declined at a faster rate than normal, suggesting increased hepatic synthesis and discharge of lipid, with retention in the plasma (7). At 2 days after antikidney serum, rat liver slices were found to incorporate [1-^{14}C] acetate into cholesterol at twice the normal rate, but by the sixth day in vitro liver acetate incorporation into both cholesterol and total fatty acids were below normal (8,9).

Most of the studies described above have been performed at a relatively early time interval after administration of either purine aminonucleoside or antikidney serum and represent an acute nephrotic state. Since most lipid studies in humans with nephrotic syndrome are done at a relatively chronic stage of the disorder, the present study of the plasma and tissue alterations in rats 3 mo following antiglomerular basement membrane induced nephrotic syndrome was undertaken to provide basic experimental data that possibly would be more comparable to the human lipid abnormalities. In addition, the specific fatty acid alterations in plasma, liver, and adipose tissue were studied in greater detail than previously.

MATERIALS AND METHODS

Wistar rats ca. 3 mo of age were given footpad injections of rabbit IgG in complete Freund's adjuvant, and 2 days later were injected intravenously with rabbit anti-rat glomerular basement membrane antiserum, using the method of Maddox et al. (10). Urinary proteins were measured by the biuret method (11), and after 12 wk, all rats with persistent urinary proteinuria greater than 40 mg/24 hr were grouped as "nephrotic"

(Group C) and those with less than this amount grouped as "nonnephrotic" (Group B). A third group of normal rats of comparable ages and weights was included as controls (Group A). After a 16 hr fast, all rats were injected with 20 μCi [1-^{14}C] acetate, sodium salt, (Amersham/Searle, Arlington Heights, IL, 52mCi/m mol) per 100 g body weight. Quantities of $^{14}CO_2$ in the expired respiratory air were measured directly and continuously for a 4 hr period using an ionization chamber with a vibrating reed electrometer and a potentiometric recorder (12). After 4 hr, rats were anesthetized with ether, and plasma, liver, and adipose tissue were obtained and stored under N_2 at-20 C until analysis. Plasma albumin was measured by an automated method using a 2(4'-hydroxyazobenzene)-benzoic acid reagent with the Technicon autoanalyzer.

Aliquots of plasmas (1.5 ml) were extracted with 10 volumes of chloroform:methanol 2:1. The combined extracts were washed once with acetate buffer (pH 4,0) to remove nonlipid components. Phospholipid concentrations were determined by digestion of aliquots of the washed total lipid extracts at 180 C for 2.5 hr with perchloric acid. Colorimetric phosphate analysis was done by addition of hydrazine sulfate, stannous chloride, and sodium molybdate, and optical densities at 815 nm recorded automatically using a Technicon Autoanalyzer.

Serum triglycerides were first extracted in N-nonane and isopropanol 2:3.5 v:v as in the method of Soloni (13). After transesterification with ethoxide, free glycerol was recovered in the aqueous phase and color was developed with acetylacetone. The absorbance was read at 410 nm in a Bausch & Lomb Model 20 colorimeter.

Total serum cholesterol was determined enzymatically as described by Allain et al. (14). Serum cholesterol esters were hydrolyzed to free cholesterol by cholesterol ester hydrolase. The total free cholesterol was then oxidized by cholesterol oxidase to cholest-4-en-3-one, with the simultaneous production of hydrogen peroxide; the latter oxidatively couples with 4-amino antipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm. A single aqueous reagent obtained from Abbott Labs (Pasadena, CA) was utilized, and after a 10 min incubation optical densities were recorded automatically using an Abbott Bichromatic analyzer.

Serum free fatty acids were methylated with diazomethane, and absolute and relative concentrations determined by gas liquid chromatography (GLC) in a Varian Model 2700 gas chromatograph using flame ionization detection and a 10% EGSS-X column at 180 C. N-pentadecanoic acid (C 15) was used as chromatographic internal standard.

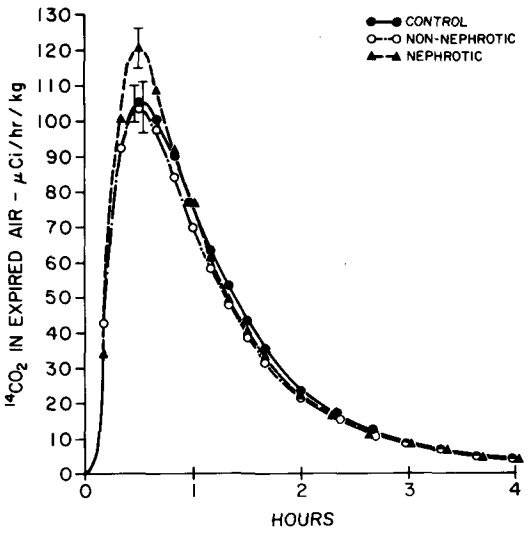

FIG. 1. Time course of $^{14}CO_2$ in expired air of normal, injected nonnephrotic and nephrotic rats.

For determinations of radioactivities, plasma lipid classes were separated by thin layer chromatography (TLC) on Silica Gel G plates coated with fluorescein. The plates were developed with petroleum ether:diethyl ether: acetic acid 80:20:1, the lipid zones detected under UV light, collected, eluted and radioactivities in aliquots of these eluates determined by liquid scintillation counting.

Liver lipids were extracted by two successive homogenizations in a total of 20X their weight of chloroform:methanol 2:1, using a Vir Tis 45 homogenizer at maximum speed for 2 min. Separation of lipid classes by TLC, quantitative analysis of each fraction, and radioactivity determinations were done as described above for plasma samples.

Total adipose tissue lipids were extracted by a procedure similar to that for liver. Fatty acid compositions of plasma and liver phospholipids, triglycerides, cholesteryl esters, and free fatty acids, and of adipose tissue total lipids were analyzed using the gas chromatographic method described above following transmethylation in BF_3-methanol.

RESULTS

Urinary 24 hr protein content of the normal control (Group A) rats was 15.8 mg ± 3.9

TABLE I

Lipid Concentrations in Plasmas of Control and Nephrotic Rats[a]

Group	Triglycerides	Cholesterol	Phospholipids	Free fatty acids
A	52 ± 7.1	71 ± 9.1	155 ± 40	32 ± 4.1
B	63 ± 6.5	81 ± 10	171 ± 54	29 ± 4.2
C	360 ± 107[b]	241 ± 81[b]	353 ± 78[b]	25 ± 6.4

[a]All values are given as mean mg/dl ± standard deviations. Group A are normal control rats (4), group B are injected nonnephrotic controls (4), and group C are nephrotic rats (4).
[b]$P<0.01$ compared to groups A and B.

TABLE II

Major Fatty Acid Compositions of Plasma Lipid Fractions of Control and Nephrotic Rats[a]

	Group	16	18	18:1	18:2	20:4
Phospholipids						
	A	18.7 ± 1.4	27.8 ± 4.6	17.6 ± 1.7	18.7 ± 1.1	17.2 ± 1.3
	B	20.3 ± 4.3	27.7 ± 3.3	16.7 ± 2.1	16.4 ± 2.0	19.9 ± 4.0
	C	18.0 ± 2.0	29.5 ± 3.0	22.7 ± 4.5	12.2 ± 5.6	17.6 ± 1.9
Free fatty acids						
	A	28.3 ± 4.4	15.2 ± 2.5	33.1 ± 4.5	17.5 ± 2.7	5.9 ± 1.3
	B	30.6 ± 5.5	13.6 ± 3.0	28.4 ± 2.8	19.2 ± 2.1	8.2 ± 2.9
	C	31.8 ± 3.1	16.6 ± 2.5	30.1 ± 6.1	13.6 ± 3.0	7.9 ± 1.8
Triglycerides						
	A	35.3 ± 5.1	4.3 ± 0.8	31.9 ± 5.7	25.5 ± 4.3	2.9 ± 0.4
	B	31.5 ± 2.8	2.3 ± 1.3	36.7 ± 4.8	26.2 ± 2.0	3.3 ± 1.2
	C	22.2 ± 4.7[b]	2.6 ± 0.5	54.3 ± 4.7[b]	16.9 ± 2.6[b]	4.0 ± 1.1
Cholesteryl esters						
	A	13.2 ± 1.4	2.4 ± 1.4	8.3 ± 1.1	18.7 ± 1.8	57.4 ± 6.0
	B	13.2 ± 2.9	4.5 ± 1.0	10.6 ± 1.4	13.2 ± 1.2	58.5 ± 5.1
	C	14.5 ± 2.1	2.5 ± 0.7	9.4 ± 0.9	19.8 ± 3.2	53.8 ± 7.4

[a]Percentage of each fatty acid ± standard deviations. For explanation of Groups A, B, and C see Table I.
[b]$P<0.01$ compared to groups A and B.

(S.D.) (Range 9-20), that of the injected nonnephrotic rat group (B) was 22.5 ± 10.1 (Range 8-36), and that of the nephrotic group (C) was 225 ±242 (Range 68-643). Total $^{14}CO_2$ in the expired air as measured by the ionization chamber method over the 4 hr period was 70.3% ± 3.6 of the injected dose in group A, 72.6% ± 5.8 in group B, and 66.8% ± 7.7 in group C; these values show no statistically significant differences between groups. The time course of $^{14}CO_2$ production in these three groups is shown in Figure 1. Metabolism of the [1-^{14}C] acetate to $^{14}CO_2$ was maximal at 30 min. No significant differences between groups were observed at any of the time intervals studied.

Serum albumin concentrations were 3.2 g/dl ± 0.2 in group A, 3.1 g/dl ± 0.3 in group B and 2.3 g/dl ± 0.4 in Group C. All plasmas of group C only were observed to be lipemic.

Mean plasma lipid concentrations in these groups are indicated in Table I. Triglyceride, total cholesterol, and phospholipid concentrations were all significantly higher in group C than in either group A or B ($P<0.01$ for all). No differences in plasma free fatty acid concentrations were observed between these groups. As seen in Table II, the increased plasma triglycerides of group C rats contained a higher proportion of oleic acid and relatively less linoleic and palmitic acids than did triglycerides of either groups A or B ($P<0.01$). There were no significant differences in fatty acid composition of plasma phospholipids, cholesteryl esters, or free fatty acids between any of the groups. Specific radioactivities, reflecting the extent of incorporation of [1-^{14}C] acetate into each of the plasma lipid classes at the end of the 4 hr period, are indicated in Table III. Group C showed increased [1-^{14}C] acetate incorporation into both phospholipids and triglycerides as compared to groups A and B ($P<0.01$ for both). No significant intergroup differences were noted in incorporation of [1-^{14}C] acetate into the free cholesterol, esterified cholesterol, or free fatty acid fractions.

Concentrations and radioactivities of the individual liver lipids in each of the experi-

TABLE III

Specific Radioactivities of Plasma Lipid Fractions in Control and Nephrotic Rats[a]

Group	A	B	C
Phospholipids	489 ± 30	552 ± 64	714 ± 76[b]
Free cholesterol	354 ± 49	304 ± 81	296 ± 88
Free fatty acids	1189 ± 140	1030 ± 183	1234 ± 208
Triglycerides	339 ± 41	322 ± 57	475 ± 60[b]
Cholesteryl esters	390 ± 70	364 ± 81	312 ± 43

[a]dpm/mg lipid ± standard deviation. For explanation of groups A, B, and C see Table I.
[b]$P<0.01$ compared to groups A and B.

TABLE IV

Lipid Concentrations and Incorporation of [1-^{14}C] Acetate into Liver Lipids of Control and Nephrotic Rats[a]

	Group	Phospholipids	Free cholesterol	Free fatty acids	Triglycerides	Cholesteryl esters
Concentration						
	A	30.7 ± 2.5	0.9 ± 0.5	0.57 ± 0.07	11.6 ± 2.7	1.9 ± 0.7
	B	29.4 ± 2.0	0.8 ± 0.3	0.53 ± 0.05	10.9 ± 2.4	1.8 ± 1.0
	C	29.1 ± 3.8	1.0 ± 0.2	0.44 ± 0.08[b]	5.8 ± 1.0[c]	1.8 ± 0.8
Total radioactivity						
	A	13,137 ± 1,802	369 ± 45	356 ± 82	16,212 ± 1,115	701 ± 73
	B	12,420 ± 1,580	315 ± 56	463 ± 69	13,332 ± 1,591	774 ± 85
	C	12,076 ± 1,645	285 ± 66	379 ± 63	4,125 ± 1,177[c]	686 ± 90
Specific activity						
	A	428 ± 81	410 ± 59	757 ± 102	1,398 ± 148	369 ± 45
	B	422 ± 50	394 ± 70	874 ± 99	1,223 ± 184	430 ± 51
	C	415 ± 44	285 ± 75	861 ± 73	711 ± 168[c]	381 ± 48

[a]Lipid concentrations are given as mg/g wet wt. liver, total radioactivity as dpm/g liver, and specific activity as dpm/mg lipid. For explanation of group A, B, and C see Table I.
[b]$P<0.05$ as compared to groups A and B.
[c]$P<0.01$ as compared to groups A and B.

mental groups are shown in Table IV. Triglyceride concentrations in group C were lower than in groups A and B ($P<0.01$) and free fatty acids were also slightly lower ($P<0.05$). Incorporation of [1-^{14}C] acetate into triglycerides of group C was decreased in comparison to incorporation into liver triglycerides of groups A and B ($P<0.01$). No intergroup differences in [1-^{14}C] acetate incorporation into any of the other lipid classes were noted. Fatty acid compositions of the liver phospholipid, triglyceride, cholesteryl ester, and free fatty acid fractions are indicated in Table V. Triglycerides of group C livers showed relatively higher percentages of oleic acid and lower arachidonic acid as compared to the two other groups ($P<0.01$).

Adipose tissue total lipid radioactivity and fatty acid compositions are indicated in Table VI. Incorporation of [1-^{14}C] acetate was higher into adipose tissue lipids of group C as compared to groups A and B ($P<0.01$). Group C showed a small decrease in percentage of palmitic acid relative to the other two groups ($P<0.05$).

DISCUSSION

In those rats in whom proteinuria persisted over the 3 mo period, plasma phospholipid, cholesterol, and particularly triglyceride concentrations remained elevated above normal control levels, although the mean plasma triglyceride concentrations of 360 mg/dl were much lower than the 3500 mg/dl previously observed in acutely nephrotic rats (5). These chronic elevated plasma triglycerides appeared also to differ qualitatively from normal plasma triglycerides in that they consisted of greater proportions of oleic acid containing species. In previous studies of early aminonucleoside induced nephrotic syndrome, plasma free fatty acid levels have been reported as either increased (2) or unchanged (1). As in the latter study, free fatty acid levels in the chronic nephrotic rats of the present study did not appear to be elevated, suggesting that if increased mobilization of fatty acids from adipose tissue sites does occur, as has been previously reported (1), that these fatty acids are rapidly esterified.

TABLE V

Major Fatty Acid Compositions of Liver Lipid Fractions of Control and Nephrotic Rats[a]

	Group	16	18	18:1	18:2	20:4
Phospholipids						
	A	19.0 ± 1.1	33.2 ± 3.2	7.3 ± 1.0	15.7 ± 1.2	24.8 ± 3.0
	B	21.6 ± 3.2	39.4 ± 6.6	4.4 ± 1.8	16.7 ± 2.6	17.9 ± 1.1
	C	20.8 ± 2.0	37.2 ± 4.5	6.6 ± 1.3	14.8 ± 1.5	20.6 ± 1.9
Free fatty acids						
	A	41.8 ± 4.0	21.6 ± 3.3	16.6 ± 2.6	11.1 ± 2.2	8.9 ± 1.1
	B	36.4 ± 4.3	31.0 ± 2.7	17.0 ± 1.8	4.7 ± 2.0	10.4 ± 2.9
	C	33.4 ± 7.2	30.2 ± 4.8	12.5 ± 1.7	9.3 ± 1.1	14.6 ± 3.4
Triglycerides						
	A	38.0 ± 4.1	3.3 ± 0.6	30.7 ± 2.7	20.1 ± 2.8	8.0 ± 1.9
	B	39.2 ± 2.9	4.5 ± 0.8	24.4 ± 3.2	23.6 ± 4.2	8.3 ± 2.8
	C	28.7 ± 4.4	4.7 ± 0.7	49.0 ± 4.6[b]	19.1 ± 2.1	2.6 ± 1.3[b]
Cholesteryl esters						
	A	71.9 ± 6.9	8.3 ± 0.7	9.2 ± 1.5	5.7 ± 1.3	5.0 ± 1.2
	B	68.4 ± 4.3	15.4 ± 1.6	6.6 ± 1.6	5.4 ± 2.1	4.2 ± 2.4
	C	70.4 ± 7.8	10.6 ± 1.2	8.5 ± 1.8	3.8 ± 1.4	6.7 ± 1.9

[a]For explanation of table see Tables I and II.

[b]$P<0.01$ as compared to groups A and B.

TABLE VI

Incorporation of [1-^{14}] Acetate and Major Fatty Acid Composition of Adipose Tissue from Control and Nephrotic Rats[a]

Group	A	B	C
Total radioactivity	15,277 ± 2,658	19,884 ± 2,633	27,650 ± 3,342[b]
Fatty acids			
16	33.3 ± 3.7	26.7 ± 4.7	22.7 ± 4.3[c]
16:1	5.4 ± 1.5	4.2 ± 1.0	6.0 ± 2.1
18	2.5 ± 0.3	5.5 ± 2.2	4.6 ± 1.0
18:1	35.3 ± 2.3	43.0 ± 6.4	37.6 ± 4.1
18:2	21.4 ± 4.2	14.5 ± 2.1	24.8 ± 3.5
18:3	2.1 ± 0.3	6.1 ± 1.6	4.3 ± 1.2

[a]Total radioactivity is given as dpm/g wet wt adipose tissue ± standard deviations. Fatty acids are given as percentages. For explanation of groups A, B, and C see Table I.

[b]$P<0.01$ as compared to groups A and B.

[c]$P<0.01$ as compared to group A.

Liver triglyceride concentrations early in the course of experimental nephrotic syndrome in rats have been variously reported to be increased (7), decreased (15,16), or unchanged (6). In vitro incorporation of [1-^{14}C] acetate into cholesterol by liver slices is increased 2 days after administration of antikidney serum, but at 6 days incorporation into cholesterol as well as total liver fatty acids falls below normal (8,9). In the present more longterm study, liver triglyceride concentration was much lower than normal, and after [1-^{14}C] acetate administration, liver triglyceride specific radioactivity was moderately reduced as compared to control groups, suggesting that hepatic triglyceride synthesis in this model of chronic experimental nephrotic syndrome is depressed below normal. The specific radioactivities of plasma triglycerides, however, were higher than normal, and incorporation of [1-^{14}C] acetate into adipose tissue lipids in the nephrotic group was also greater than normal. These results suggest that, in the present chronic nephrotic rat, the major source of the increased plasma triglycerides may be fatty acids mobilized from adipose tissue stores. If these adipose tissue fatty acids are esterified by the liver, this esterification possibly occurs in a pool with a turnover rate different from that of the liver triglycerides formed directly in the liver from the administered acetate. Incorporation of [1-^{14}C] into plasma and liver cholesterol was not significantly increased, which is consistent with earlier studies indicating that cholesterol synthesis is increased in the early but not later stages of experimental nephrosis (19,20).

TABLE VII

Comparison of Changes Observed in the Acute vs. Chronic Nephrotic Syndrome[a]

	Acute	Chronic
Serum albumin	− − −	−
Urinary protein	+ + +	+ +
Plasma triglyceride concentration	+ + + +	+ +
[^{14}C] acetate	+	+
Plasma cholesterol concentration	+ + +	+
[^{14}C] acetate	+	0
Liver triglyceride concentration	+ 0 −	− −
[^{14}C] acetate	+	−
Liver cholesterol concentration	+ 0	0
[^{14}C] acetate	+ −	0

[a]Notations in the "acute" column are estimations of changes during the first 2 wk of the nephrotic syndrome and were compiled from all the references described in the text. The "Chronic" column refers to the present 3-mo duration experiments as compared to the "acute" data of others. Plus (+) symbols refer to increases, (−) to decreases, and (0) to no change. Where conflicting results have been reported, these are all included (e.g. + 0 −).

Since oxidation of the administered [1-^{14}C] acetate to $^{14}CO_2$ was not significantly different between the three experimental groups, it is unlikely that the observed differences in [1-^{14}C] acetate incorporation into the various lipid fractions were attributable to differences in availability of substrate.

In Table VII, the magnitude of some of the changes in the acute nephrotic syndrome as compiled from data of all the studies referred to above have been compared to the data derived from the present chronic nephrotic syndrome experiments. In summary, in the chronic nephrotic syndrome, serum albumin, urinary protein, and plasma triglyceride and cholesterol concentration alterations are less marked than those observed in the acute state. Incorporation of acetate into plasma triglycerides is increased in both the acute and chronic nephrotic syndrome. Liver triglyceride concentration is variable in the acute nephrotic syndrome and decreased in the chronic state. Incorporation of acetate into liver triglycerides appears to be increased in the acute state but was decreased in the chronic experiments.

Synthesis and turnover of plasma triglycerides in human chronic nephrotic syndrome is increased (1,21); these observations are consistent with our present data regarding plasma triglycerides in chronic experimental rat nephrotic syndrome. Further studies will be required to determine whether in chronic rat nephrotic syndrome the clearance of plasma triglycerides and the activity of plasma lipoprotein lipase are also decreased, as has been found in some studies of human nephrotic syndrome (1).

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The Effects of Original and Randomized Rapeseed Oils Containing High or Very Low Levels of Erucic Acid on Cardiac Lipids and Myocardial Lesions in Rats

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ABSTRACT

The nutritional status of the very low-erucate rapeseed oil, *Brassica napus* var. 'Tower,' was compared with that of the high-erucate oil, *Brassica napus* var. 'Target,' as well as with corn oil. The effect of randomization on the nutritional qualities of rapeseed oil was investigated as well. The feeding of diets containing the original and randomized 'Tower' oil or the original 'Target' oil, at the 20% level by weight, gave growth rates which were not significantly different from that for corn oil. However, the randomized 'Target' oil gave growth rates which were significantly less than all other groups. The growth results could not be explained simply on the basis of food consumption. The level of triglycerides in the hearts of rats fed the very low-erucate oils was not significantly different from the corn oil group. Triglyceride concentrations in the hearts of animals given the high-erucate oils were 7-12 times greater than all other groups. The level of total fatty acids in tissue phospholipids was the same regardless of dietary treatment. Fatty acid compositions of the tissue lipids were the same in animals fed either the original or randomized rapeseed oils. A much higher incidence of focal myocardial necrosis was found in animals receiving high-erucate rapeseed oil relative to animals given the corn oil. The incidence in rats fed diets containing very low-erucate rapeseed oil was intermediate between these latter two extremes.

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TABLE I

Composition of Diets

Ingredients	Weight % of total
Casein (vitamin-free)[a]	20.0
Dextrose	50.5
Oil[b]	20.0
Alpha-floc	5.0
Salt mixture[c]	3.5
Vitamin mixture[d]	1.0

[a]General Biochemicals Inc., Chagrin Falls, OH.

[b]Either corn oil (CO), original Tower rapeseed oil (LEO), randomized Tower rapeseed oil (LER), original Target rapeseed oil (HEO), or randomized Target rapeseed oil (HER).

[c]Williams-Briggs modified, General Biochemicals Inc., location pls.

[d]Supplied the following in mg/kg of diet: retinyl palmitate, 20; ergocalciferol, 0.025; dl-α-tocopheryl acetate, 200; menadione, 1; thiamine HCl, 10; riboflavin, 10; pyridoxine HCl, 10; pantothenate, 20; biotin, 0.2; folic acid, 1; nicotinic acid, 50; choline dihydrogen citrate, 1,500; inositol, 100; vitamin B_{12} (0.1% trituration), 20.

INTRODUCTION

The adverse effects of feeding diets containing rapeseed oil with high levels of erucic acid to rats have been well documented (1-12). These effects include a depression in growth (1-5), an early accumulation of cardiac lipid (6-10), as well as a high incidence of myocardial necrosis (9-12). With the recent availability of rapeseed oils containing very low levels of erucic acid, it is now of interest to compare such oils with the older varieties.

The purpose of the experiments described herein was to evaluate the effect of *Brassica napus* var. 'Tower' rapeseed oil having less than 0.5% erucic acid on the growth, food consumption, cardiac lipid composition, and histopathology of heart tissue. This latter oil was compared to *Brassica napus* var. 'Target' rapeseed oil which contained erucic acid at the level of 34% and to corn oil. In addition, the influence of the positional distribution of the fatty acids in the dietary triglycerides on growth and the myocardial alterations was tested by feeding fully randomized rapeseed oils.

MATERIALS AND METHODS

Animals and Diets

Male Sprague-Dawley rats weighing ca. 50 g were obtained from the Bio-Breeding Laboratories (Ottawa, Ont.). They were housed individually in stainless steel cages and fed Purina

TABLE II

Fatty Acid Composition of Dietary Oils[a]

Fatty acid	CO	LEO	LER	HEO	HER
	Weight % of total				
14:0	tr[b]	tr	tr	tr	tr
16:0	12.3	2.5	5.0	3.8	3.7
16:1	tr	tr	tr	tr	tr
18:0	1.9	2.3	2.0	1.7	1.6
18:1	25.2	55.0	56.2	26.3	25.8
18:2	60.0	28.3	27.1	16.6	16.9
18:3	0.5	9.7	8.1	6.7	7.7
20:0	tr	0.6	0.6	0.7	0.6
20:1	tr	1.2	1.0	10.0	10.0
22:1	–	tr	tr	34.2	33.7
Other	tr	tr	tr	tr	tr

[a]Abbreviations as given in footnote to Table I.
[b]Tr indicates trace (<0.5% of the total).

TABLE III

Fatty Acid Composition of 2-Monoglycerides Derived from Rapeseed Oil Triglycerides[a]

Fatty acid	LEO	LER	HEO	HER
	Weight % of total			
14:0	tr	tr	tr	tr
16:0	0.6	7.3	0.7	4.2
16'1	tr	tr	tr	tr
18:0	0.3	2.2	0.4	2.0
18:1	45.7	56.7	40.3	26.2
18:2	37.8	24.9	38.9	16.4
18:3	14.4	8.0	16.5	6.8
20:0	tr	tr	tr	0.8
20:1	0.6	0.7	2.4	10.3
22:1	tr	tr	1.0	33.3
Other	0.6	tr	tr	tr

[a]The 2-monoglycerides were isolated following hydrolysis of dietary triglycerides with pancreatic lipase. Abbreviations identified in footnote to Table I.

Lab Chow (Ralston-Purina Co., St. Louis, MO) and water ad libitum for 8 days. They were then randomized into five experimental groups of 30 animals each. Each group was fed a semipurified diet as described in Table I which contained corn oil, original or fully randomized 'Tower' rapeseed oil (<0.5% erucic acid), original or fully randomized 'Target' rapeseed oil (34% erucic acid) as the test fat at the 20% level. The fully refined 'Tower' and 'Target' rapeseed oils were provided by the Rapeseed Association of Canada. The randomized oils were prepared by Canada Packers Ltd. (Toronto, Ont.). The body weights and food consumption were recorded twice weekly throughout the feeding period which lasted up to 16 wk. All diets were stored under refrigeration. A second experiment was conducted on a much smaller scale in which rats (five per treatment) were maintained on diets containing corn oil, original 'Tower' rapeseed oil, or fully randomized Tower rapeseed for the purpose of lipid analyses after 3 days.

Lipid Analyses

Five rats were sacrificed from each group for analyses of cardiac lipids after feeding the experimental diets for periods of 3 and 7 days, respectively. After subjecting the animals to cervical fracture, the hearts were rapidly excised, rinsed in cold 0.25 M sucrose solution containing 50 mM Tris-HCl (pH 7.4), and weighed. They were immediately homogenized in 10 volumes of the iced sucrose-Tris buffer using a Potter-Elvehjem homogenizer with a Teflon pestle. Total lipid extracts were prepared from the heart homogenates (13) after the addition of tripentadecanoin as an internal standard. The triglycerides and phospholipids were separated by thin layer chromatography and their derived fatty acid methyl esters were quantitated by gas liquid chromatography in the presence of methyl pentadecanoate as described elsewhere (14). The fatty acid composition of the 2-monoglycerides derived from the dietary triglycerides was also determined following hydrolysis with pancreatic lipase (15).

Histopathological Examination

Animals were killed by chloroform overdose and, after necropsy, small pieces of ventricular walls from each rat heart were frozen by means of liquid nitrogen and sectioned with a cryostat for Oil red O staining. The heart tissues were cut into sizes of less than 1 mm^3 and fixed in 2.5% buffered glutaraldehyde, postfixed in 1.0% osmium tetroxide, and embedded in Epon-812. The Epon blocks were sectioned at 1

TABLE IV

Body Weight and Cumulative Food Consumption of Rats at Various Experimental Periods[a]

Time on diets	CO	LEO	LER	HEO	HER
14 days					
Body weight, g	174 ± 3[a]	177 ± 3[a]	174 ± 3[a]	171 ± 3[a]	162 ± 3[b]
Cumulative food consumption, g	194 ± 5[a]	203 ± 6[ab]	213 ± 6[b]	188 ± 4[a]	190 ± 4[a]
56 days					
Body weight, g	355 ± 6[ab]	362 ± 4[a]	353 ± 3[ab]	342 ± 5[b]	325 ± 7[c]
Cumulative food consumption, g	862 ± 17[a]	910 ± 13[b]	928 ± 21[b]	860 ± 10[a]	838 ± 12[a]

[a]All values are given as means ± S.E. for 20 rats in each group. Means with different superscript letters are significantly different from each other ($P<0.05$) as judged by analysis of variance and Duncan's multiple range test (16). The initial body weight of rats was 91 ± 1 g prior to feeding the semipurified diets. Abbreviations are identified in footnote to Table I.

TABLE V

Effect of Dietary Treatments on Level of Fatty Acids in Heart Triglycerides[a]

Time on diets	CO	LEO	LER	HEO	HER
	mg/g heart				
Experiment 1					
3 days	3.4 ± 0.2[a]	2.8 ± 0.2[a]	2.0 ± 0.2[a]	23.7 ± 2.8[b]	24.2 ± 0.9[b]
7 days	2.0 ± 0.2[a]	2.0 ± 0.4[a]	2.8 ± 0.3[a]	24.0 ± 4.8[b]	20.9 ± 2.9[b]
Experiment 2					
3 days	4.6 ± 0.6[a]	4.3 ± 0.3[a]	4.4 ± 0.8[a]		

[a]All values are given as means ± S.E. for five rats in each group and represent mg of fatty acids in triglycerides per g of heart. See also footnote to Table IV. The mean heart weights for all groups ranged from 0.55 to 0.67 g and were not signifiantly different from each other ($P<0.05$). The initial body weight of rats in Experiment 2 was 106 ± 2g prior to feeding the semipurified diets. Abbreviations are identified in footnote to Table I.

μ thickness and stained with toluidine blue. The remaining portion of the heart was fixed with 10% calcium formalin and trimmed longitudinally so that the sections would include four cardiac chambers. The tissues fixed in formalin were processed for routine haematoxylin and eosin staining. All histopathological examination of myocardial sections were conducted on a blind basis without information on the dietary treatments.

RESULTS

Table II gives the fatty acid composition of the different oils used in the present experiments. Linoleate was the major acid in the corn oil whereas it represented only 28 and 17% of the total acids in the low and high erucate oils, respectively. The rapeseed oils had significant quantities of linolenic acid (7-10%); the low and high erucate oils were quite high in oleic and erucic acids, respectively. Randomization had no significant effect on the fatty acid composition of the rapeseed oils. Comparison of the data in Tables II and III indicates that the linoleic and linolenic acids were preferentially associated with the 2-position of the original rapeseed oil triglycerides whereas the erucate in the original 'Target' rapeseed oil (HEO) was almost completely excluded from this position. However, the fatty acid compositions of the 2-monoglycerides derived from the randomized 'Tower' rapeseed oil (LER) and randomized 'Target' rapeseed oil (HER) (Table III) are almost identical to those of the corresponding triglycerides (Table II) which indicates that randomization was complete.

The rate of weight gain among the experimental groups was significantly different ($P<0.05$) throughout the period of 16 wk. As early as the end of the second week, the body weight of rats fed the HER diet was less than that for all other groups (Table IV). This reduction in growth rate was also apparent at 8 wk

TABLE VI

Effect of Dietary Treatments on Level of Fatty Acids in Heart Phospholipids[a]

Time on diets	CO	LEO	LER	HEO	HER
	mg/g heart				
Experiment 1					
3 days	11.4 ± 0.6	11.0 ± 1.1	11.0 ± 0.8	9.7 ± 1.0	10.7 ± 1.8
7 days	11.7 ± 0.4	12.1 ± 0.6	11.0 ± 0.6	12.1 ± 0.9	12.1 ± 0.6
Experiment 2					
3 days	9.7 ± 0.6	11.0 ± 0.6	11.0 ± 0.9		

[a]No significant difference was found in the level of fatty acids in heart phospholipids between dietary groups by analysis of variance. The values given in the table include trace amounts of alk-1-enyl-and alkyl-glyceryl ethers (17). See also footnotes to Table V. Abbreviations are identified in footnote to Table I.

TABLE VII

Fatty Acid Composition of Heart Triglycerides after Feeding Experimental Diets for Seven Days[a]

Fatty acid	CO	LEO	LER	HEO	HER
	weight % of total				
16:0	28.0 ± 2.3	19.1 ± 1.5	19.3 ± 0.4	4.5 ± 1.4	7.1 ± 0.6
16:1	3.1 ± 0.3	3.1 ± 1.0	5.0 ± 0.4	1.1 ± 0.1	1.2 ± 0.1
18:0	8.9 ± 0.7	7.0 ± 0.7	6.5 ± 0.5	2.7 ± 0.1	3.1 ± 0.1
18:1	28.7 ± 0.8	45.0 ± 1.7	46.0 ± 1.1	26.2 ± 1.8	28.2 ± 0.8
18:2	21.6 ± 4.0	12.5 ± 2.3	15.4 ± 1.4	10.3 ± 0.7	9.9 ± 0.4
20:1	1.5 ± 0.5	2.3 ± 0.6	1.5 ± 0.2	13.3 ± 0.4	13.5 ± 0.2
22:1	tr	tr	tr	34.2 ± 2.9	31.9 ± 1.7
S[b]	36.9	26.1	25.8	7.2	10.2
U[b]	54.9	62.9	67.9	85.1	84.7
S/U	0.67	0.42	0.38	0.09	0.12

[a]Fatty acids not contributing at least 3.5% to the total in any one group have been omitted from the table. Student's t test (16) showed that the weight percentages of the individual fatty acids in the original versus the randomized groups were not significantly different. Abbreviations are identified in a footnote to Table I.

[b]S, sum of major saturated fatty acids; U, sum of major unsaturated fatty acids.

and later and could not be attributed to differences in food consumption. The animals in the original 'Tower' rapeseed oil (LEO) and LER groups grew as well as those in the corn oil (CO) group.

The rats fed LEO or LER had triglyceride concentrations in heart which were not significantly different from those animals fed CO for 3 or 7 days (Table V). The level of triglycerides in the HEO and HER groups was 7-12 times that for the CO controls. The slightly higher concentrations of cardiac triglycerides in Experiment 2 as compared to Experiment 1 may be due in part to the fact that these two experiments were conducted at different times of the year and to the use of somewhat older and larger animals in the latter experiment. In contrast to the heart triglycerides, the nature of the dietary oil had no significant effect on the concentration of the phospholipids (Table VI).

The fatty acid compositions of heart triglycerides are given in Table VII following 7 days on the diets. The percentage of oleic acid in the heart triglycerides could be arranged in order of decreasing magnitude as LEO = LER > CO = HEO = HER which agrees with the relative abundance of this same acid in the corresponding oils (Table II). Also, the percentage of linoleic acid in the tissue triglycerides showed CO > LEO = LER > HEO = HER which agreed with the order based on the fatty acid composition of the dietary oils. One-third of the fatty acids in the heart triglycerdes from the HEO or HER groups were represented by erucic acid. Interestingly, palmitic acid contributed 19% to the total fatty acids in the tissue triglycerides from the LEO and LER groups but only 5-7% in the HEO and HER groups even though this acid represented ca. 4% of the fatty acids in all four of these dietary oils. However, it can be calculated from the data in Tables V and VII that the absolute amounts of palmitate in heart

TABLE VIII

Fatty Acid Composition of Heart Phospholipids after Feeding Experimental Diets for Seven Days[a]

Fatty acid	CO	LEO	LER	HEO	HER
	weight % of total				
16:0	12.5 ± 0.4	10.2 ± 0.3	11.0 ± 0.5	8.1 ± 0.2	7.7 ± 0.2
18:0	25.9 ± 0.4	24.8 ± 0.7	24.6 ± 0.6	22.0 ± 0.7	22.6 ± 0.6
18:1	7.1 ± 0.2	13.8 ± 0.5	13.0 ± 0.3	12.5 ± 0.3	13.1 ± 0.3
18:2	21.4 ± 1.2	17.8 ± 0.8	18.0 ± 0.4	18.4 ± 0.5	16.9 ± 0.8
20:1	tr	0.6 ± 0.1	0.6 ± 0.2	3.8 ± 0.2	3.8 ± 0.1
20:4	20.5 ± 0.8	18.4 ± 0.4	20.8 ± 0.2	19.1 ± 1.0	21.0 ± 0.6
22:1	tr	tr	tr	3.5 ± 0.3	3.6 ± 0.3
22:6	7.5 ± 0.3	8.5 ± 0.8	8.4 ± 0.5	6.3 ± 0.6	6.6 ± 0.5
S	38.4	35.0	35.6	30.1	30.3
U	56.5	59.1	60.8	63.6	65.0
S/U	0.68	0.59	0.59	0.47	0.47

[a]See footnote to Table VIII. Abbreviations are identified in footnote to Table I.

triglycerides (mg/g tissue) in the HEO and HER treatments are two- to four-fold greater than for the other three groups. The fatty acid compositions were not significantly different for the original vs. the randomized rapeseed oils. The ratio of saturated/unsaturated fatty acids in the groups fed high-erucate oils was much less than that for the animals fed corn oil whereas the ratio for the very low-erucate groups was intermediate between these two extremes.

In direct contrast to the triglyceride data, the fatty acid composition of the heart phospholipids was not drastically influenced by the type of dietary oil (Table VIII). Thus, the percentages of stearic, linoleic, arachidonic, and docosahexaenoic acids were relatively similar in the tissue phospholipids from all five groups. The somewhat higher percentage of saturated fatty acids in the very low-erucate as compared to the high-erucate groups was accompanied by the presence of small amounts of erucic acid in the latter. The tissue phospholipids from all dietary groups had greater percentages of stearic, arachidonic, and docosahexaenoic acids than did the corresponding triglycerides while the converse was true for oleic acid. A preferential entry of erucic acid into cardiac triglycerides relative to phospholipids as observed in this study has been reported previously (9,18).

Pathological investigations after 16 wk of feeding the various diets showed no significant macroscopical lesions in any organs of all rats. None of the frozen and 1 μ sections showed accumulations of lipid droplets in cardiac muscle fibres. Focal areas of myocardial necrosis reaching approximately 0.4 x 0.3 mm in size were observed in some animals fed the experimental diets (Table IX). These lesions

TABLE IX

Effect of Dietary Treatments on Number of Rats Showing Focal Myocardial Necrosis[a]

	Number of animals Examined	Number of animals showing lesions[b]
CO	15	0
HEO	15	6
HER	15	3
LEO	15	3
LER	15	2

[a]Animals were maintained on diets for 16 wk. Abbreviations are identified in footnote to Table I.

[b]Hearts showed a few small foci of myocardial necrosis in the longitudinal sections.

were most frequently located in the myocardium beneath the epicardium of the left ventricular wall. Fresh lesions of the myocardial necrosis (Fig. 1) were characterized by hyaline degeneration and sarcolysis of the cardiac muscle fibres accompanied by mesenchymal cell reaction. Relatively old lesions (Fig. 2) exhibited an infiltration of lymphoid elements, macrophages, and fibroblasts which replaced the degenerated muscle fibres.

DISCUSSION

The present study was initiated so as to investigate the effect of feeding diets containing rapeseed oil with only trace levels of erucic acid, as well as the effect of randomization of rapeseed oils, on the composition of cardiac lipids and the development of myocardial lesions in the rat. No early elevation in triglyceride levels relative to controls given CO was observed with LEO or LER ('Tower' oils).

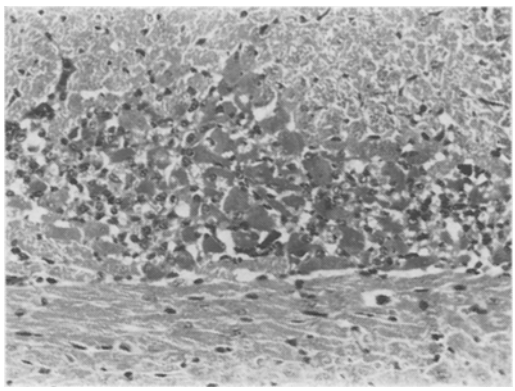

FIG. 1. Fresh foci of myocardial necrosis with mesenchymal cell reaction from heart tissue of a rat fed original 'Tower' rapeseed oil (LEO). Stained with haematoxylin and eosin. Magnification, 319X.

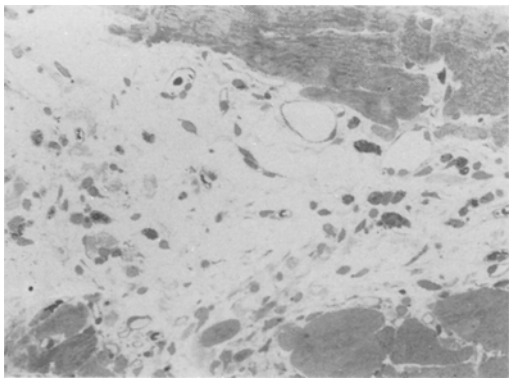

FIG. 2. Relatively old foci of myocardial necrosis. Muscle fibres were lost and a small number of fibroblasts and macrophages were scattered in the foci. Heart tissue from a rat fed original 'Target' rapeseed oil (HEO). Stained with Toluidine blue. Magnification, 319X.

The very pronounced accumulation of triglyceride in heart tissue after 3 or 7 days of feeding diets containing the high-erucate oils ('Target') was not significantly different in magnitude for HEO or HER. However, a suppression in growth was observed with HER relative to HEO. This latter growth phenomenon may reflect an increased absorption of erucic acid, in the form of 2-monoerucin, when HER is fed as previously suggested (5). However, the failure of HER to produce an even greater level of cardiac triglycerides than HEO, and the almost identical fatty acid composition of cardiac lipids from both these groups, was not consistent with expectations based on this hypothesis. It is possible, however, that differences in erucate content of lipids from other tissues may have existed between animals fed HEO or HER. Our results differ from those of Rocquelin (19) who found that the high erucate rapeseed oil gave total lipid and erucate levels in heart which were slightly higher when the oil was interesterified. The differences in results may be due to the use of rapeseed oil in Rocquelin's work which contained much higher levels of erucate than employed in our studies. In addition, this former worker used Wistar rats whereas we used Sprague-Dawley.

Our results are in general agreement with those of others which indicate that the content of erucic acid in the dietary oil does correlate fairly well with the degree of cardiac lipidosis but not as well with the incidence of myocardial lesions. Thus, Beare-Rogers et al. (20) have shown that the dietary intake of a docosenoic acid was a most important factor in producing the accumulation of cardiac lipids. Furthermore, Kramer et al. (21) reported a significant elevation in levels of total heart lipids from male rats fed diets containing rapeseed oil with 22% erucic acid for 1 wk but not with rapeseed oils having 1.6 or 4.3% erucate. However, the incidence of cardiac lesions after 16 wk was identical in all three groups (21). In similar studies, Vogtmann et al. (22) have claimed that the incidence of lesions after feeding diets containing a number of different rapeseed oils (0.8, 0.9, 3.7, and 20.6% erucic acid) was similar on all treatments and no worse than soybean oil. In contrast to these latter findings, Beare-Rogers et al. (23) found that a number of rapeseed oils (1.9, 3.4, and 23.3% erucic acid) gave a significantly higher incidence of cardiac lesions than the incidence for those in control animals receiving soybean oil. As reported herein, a much lower incidence of myocardial necrosis was observed in rats receiving the 'Tower' ($<$0.5% erucate) as compared to 'Target' (34% erucate) rapeseed oil. Recently, Vles et al. (24) reported a higher incidence of lesions in hearts of rats fed regular rapeseed oil (34% erucate) as compared to those fed either a rapeseed oil containing 1.0% erucic acid or sunflowerseed oil.

Our present results, and some of those discussed above, suggest that factors other than erucic acid may contribute to the development of myocardial necrosis in rats since the number of animals showing lesions with the very low-erucate oils was approximately midway between that found for the corresponding high-erucate oils and corn oil (Table IX) even though the 'Target' oils contain 70 times more erucic acid than the 'Tower' oils. In this regard, it has been suggested that the nutritional characteristics of rapeseed oils may be related to the relative abundance of saturated and unsaturated

fatty acids in these oils (10,20,25). Furthermore, it has been postulated that linolenic acid plays a role in the etiology of cardiac necrosis when rats are fed diets containing low-erucate rapeseed oils (26).

It is difficult at present to explain the elevation in concentration of cardiac triglycerides with no increase in phospholipids when rats are fed high-erucate rapeseed oils since both lipids are synthesized de novo from precursor 1,2-diglycerides (27). It would appear that other metabolic alterations associated with impaired fatty acid oxidation (28-30) or an increased uptake of fatty acids by cardiac tissue (31) are also responsible for this latter phenomenon. Further experimentation will be necessary to establish the nature of these other metabolic changes.

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Origin of Fatty Acids of Cholesteryl Ester Accumulated by Fu5AH Cells in Culture

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ABSTRACT

The Fu5AH rat hepatoma cell line accumulates cholesteryl ester (CE) upon incubation in medium supplemented with hyperlipemic serum or hyperlipemic serum lipoproteins. This cell line was used to investigate the origin of the fatty acids esterified to cholesterol in intracellular accumulations of CE. The intracellular CE-fatty acid distribution was found to be markedly different from that of the lipoprotein which stimulated the accumulation. Free fatty acids added to the culture medium were found esterified to cholesterol in the cells, demonstrating that cellular esterification contributes to the accumulation of CE. Using a subline of Fu5AH cells containing radioactively labeled intracellular fatty acids, it was found that about one-third of the fatty acid moiety of CE accumulated by the cells during a 24 hr incubation with hyperlipemic serum was derived from endogenous fatty acids. The drug chloroquine was found to inhibit cellular cholesterol esterification, so that only 4% of CE-fatty acids were derived from endogenous fatty acids. Evidence is presented suggesting a major role for cellular esterification in CE accumulation by Fu5AH cells.

INTRODUCTION

Cellular cholesteryl ester (CE) accumulation is a prominent event in atherosclerosis. A cell culture model system, the Fu5AH rat hepatoma cell line, has been used in our current investigations into the origin of cellular CE accumulation. This cell line was selected because of its ability to accumulate CE upon incubation in medium containing hyperlipemic serum or hyperlipemic lipoproteins, but not normal serum (1). Fu5AH cells accumulate lipid in the form of intracellular droplets (1) which are morphologically similar, by both polarizing light microscopy and scanning electron microscopy, to those found in human aortic atheromata (2-4).

In an earlier investigation, Rothblat et al. (5) reported on the origin of the cholesteryl moiety of the CE accumulated by Fu5AH cells incubated with hyperlipemic serum. In that study, it was determined that after a 24 hr incubation ca. 10% of the CE which accumulated came from endogenous free cholesterol (FC), 30% from exogenous FC, and 60% from exogenous CE. The present studies were designed to investigate the source of the fatty acids in the CE accumulated by these cells. This was accomplished by studies of CE-fatty acid composition of cells grown in medium supplemented with lipoproteins isolated from hyperlipemic serum and by studies of CE accumulation by cells whose intracellular fatty acids were isotopically labeled.

MATERIALS AND METHODS

Cells and Growth Conditions

The Fu5AH rat hepatoma cell line was derived from the Reuber H35 rat hepatoma (6-8). Stock cultures of these cells were grown in Eagle's minimal essential medium (MEM) supplemented with 10% fetal bovine serum. A subline of these cells, designated AH-DLP, was adapted to growth on a lipid deficient medium. AH-DLP cells were grown in MEM supplemented with 5 mg/ml of delipidized serum protein (9). These cells, growing in a lipid deficient medium, synthesize the lipid needed for continued growth and contain less than 1 μg/mg protein of esterified cholesterol. A subline of AH-DLP cells was maintained for more than 23 population doublings in lipid deficient medium containing [^{14}C]-U-glucose of a constant specific activity (24,000 cpm/μmole glucose) in order to label intracellular fatty acids. At the time of the first experiments reported here, the originally unlabeled cells represented less than 1×10^{-7} of the mass of the cells used in the experiments. Experiments were performed more than 19 population doublings after a steady state level of cellular total fatty acid specific activity was reached, at which point it could be assumed that all pools of intracellular fatty acids were of equal specific activity.

Serum and Serum Lipoproteins

Hyperlipemic rabbit serum was obtained

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TABLE I

Fatty Acid Composition of Cholesteryl Esters (% of total)

Fatty acid[a]	Lipoprotein (d<1.019)[b]	Control cells[c,d]	Cells incubated with lipoprotein[d,e]	Cells incubated with lipoprotein + 18:3[d,f]
16:0	18.3 ± 2.0	7.7 ± 1.3	12.7 ± 1.0	14.9 ± 4.8
16:1	4.7 ± 0.5	9.2 ± 4.5	10.0 ± 1.5	10.0 ± 0.4
18:0	3.6 ± 0.7	4.0 ± 0.9	4.3 ± 0.6	4.0 ± 1.4
18:1	37.2 ± 1.5	73.3 ± 5.4	62.7 ± 3.1	50.9 ± 2.1
18:2	31.9 ± 0.1	0.8 ± 0.4	5.6 ± 0.6	6.7 ± 4.1
18:3 + 20:1	3.0 ± 0.6	<0.1	<0.1	11.4 ± 4.9

[a]16:0 = Palmitic acid, 16:1 = Palmitoleic acid, 18:0 = Stearic acid, 18:1 = Oleic acid, 18:2 = Linoleic acid, 18:3 = Linolenic acid, 20:1 = Eicosenoic acid.

[b]Mean ± Standard Deviation (N = 3).

[c]Cells are routinely cultured in Eagle's minimal essential medium (MEM)-10% FBS.

[d]CE content (μg cholesterol/mg protein): control - 13, cells + lipoprotein - 36, cells + lipoprotein + 18:3 - 42.

[e]Lipoprotein was added to medium at a concentration of 100 μg/ml of free cholesterol (FC). Incubation time was 24 hr.

[f]Linolenic acid (18:3) was added to medium at a concentration of 50 μg/ml, bound to albumin at a concentration of 2 mg/ml. Incubation time was 24 hr.

from rabbits fed rabbit chow with 1% cholesterol and 6% corn oil added. Rabbits were fasted for 24 hr prior to bleeding. A lipoprotein fraction of d<1.019 was isolated from hyperlipemic rabbit serum by ultracentrifugation (Spinco, No. 40 rotor, Beckman Instruments, Inc., Palo Alto, CA) in KBr solutions (10). This density cut contains both the very low density lipoprotein (VLDL) fraction, d<1.006, and the intermediate density lipoprotein (IDL) fraction, d 1.006-1.019. Marked increases in the concentration of these lipoproteins have been observed in cholesterol-fed rabbits together with a change in composition resulting in an increase in the percentages of CE and a decrease in triglycerides (11,12). Lipoproteins were dialyzed extensively against 0.15M NaCl + 0.001M EDTA and against tissue culture medium prior to addition to cell cultures.

Experimental Procedures

Cell monolayers were washed twice with Grey's buffered salt solution, harvested with 0.25% trypsin in 0.1% versene, and washed twice by recentrifugation. Cell pellets were resuspended in distilled H_2O and sonicated (Branson sonifier, setting No. 1, 20 sec.). Aliquots were taken for protein determination by the method of Lowry et al. (13), and for lipid extraction by the method of Bligh and Dyer (14). The total lipid extract was separated into lipid classes by thin layer chromatography on Silica Gel G (J.T. Baker Chemical Co., Phillipsburg, NJ) with a solvent system of petroleum ether:ethyl ether:acetic acid, 80:20:1. Isolated lipid classes were eluted from the silica gel by extraction with $CHCl_3$.

Fatty acid methyl esters were prepared by the BF_3 method of Morrison and Smith (15). Gas liquid chromatographic (GLC) analysis was performed at 200 C on a Hewlett-Packard No. 402 chromatograph, using 6 ft columns packed with 10% SP-2330 on 100/120 Chromosorb W AW (Supelco, Inc., Bellefonte, PA). Fatty acid methyl esters were identified by comparison of their retention times with those of known standards purchased from Supelco, Inc. Free and total cholesterol content were determined by GLC procedures as previously described (1).

Total fatty acids were quantitated by the colorimetric method of Duncombe (16) following saponification with ethanolic KOH (17). Glucose was quantitated with glucose oxidase (Glucostat, Worthington Biochemical Corp., Freehold, NJ).

Linolenic acid was purchased from Applied Science Laboratories, Inc. (State College, PA). The purity was greater than 98% as determined by GLC analysis of the methyl ester. Linolenic acid was added to the cellular growth medium bound to fatty acid-free albumin (Miles Laboratories Co., Kankakee, IL). The sodium salt of linolenic acid was mixed with albumin in solution and allowed to equilibrate at least 30 min prior to addition to the medium. Chloroquine was purchased from Sigma Chemical Co. (St. Louis, MO).

[^{14}C]-U-glucose (150-250 mCi/mM) was purchased from the New England Nuclear Co., Boston, MA. Radioactivity in the medium was determined by counting replicate aliquots in Aquasol (New England Nuclear).

TABLE II

Incorporation of Endogenous Fatty Acids into Cellular Cholesteryl Ester (CE)

Medium supplements	Cellular CE content (μg/mg protein)	Fatty acid (FA) specific activity[a] (cpm/μmole)		% of CE-fatty acids derived from endogenous fatty acids[b]
		CE	Total lipid	
Delipidized serum protein (Control)	<1	-----	23,530	100
Hyperlipemic serum[c]	68	8,390	9,520	36
d<1.019 lipoprotein[d]	27	10,550	12,500	45
d<1.019 lipoprotein + chloroquine[e]	20	940	12,060	4

[a]Listed values are the mean of two independent determinations.

[b]CE-Fatty acid specific activity of treated cultures/total fatty acid specific activity of controls.

[c]Hyperlipemic rabbit serum added to medium at a level of 2.5%.

[d]d<1.019 lipoprotein from hyperlipemic rabbit serum added to medium at concentration of 100 μg/ml free cholesterol.

[e]40 μg/ml chloroquine.

Hyperlipemic serum was labeled with 4-[^{14}C]-cholesterol (250 mCi/mM, New England Nuclear) by addition of the isotope in ethanol (18). Ethanol concentration in the medium was kept below 0.5%. Radioactivity was determined in a Packard Tri-carb liquid scintillation spectrometer, using 0.6% PPO and 0.02% dimethyl-POPOP in toluene. The term "% esterification" refers to the percentage of labeled FC taken up by the cells that is recovered as cellular CE. The amounts of exogenous FC incorporated into cellular FC and CE were determined by dividing the cpm/mg protein in FC or CE by the specific activity (cpm/μg cholesterol) of exogenous FC (5).

In experiments with AH-DLP cells with ^{14}C-labeled intracellular fatty acids, the total fatty acid specific activity of control cells was determined in each experiment as a measure of endogenous fatty acid specific activity prior to incubation with hyperlipemic serum. Control cells contained little CE (<1 μg/mg protein). These labeled cells were then incubated for 24 hr in medium labeled with [^{14}C]-U-glucose to which was added either unlabeled hyperlipemic serum or unlabeled d<1.019 lipoprotein. These conditions stimulate CE accumulation by the AH-DLP cells. The specific activity of the fatty acids esterified to cholesterol was determined and compared with that of endogenous fatty acids at zero time to calculate the percentage contribution of endogenous fatty acids to the fatty acid moiety of the accumulated CE.

RESULTS

Cholesteryl Ester (CE) Fatty Acid Composition

Control cells, growing in MEM supplemented with 10% fetal bovine serum, contain only low levels of CE (5-15 μg/mg protein). However, upon incubation in medium containing hyperlipemic rabbit serum or d<1.019 lipoprotein isolated from hyperlipemic serum, the cells accumulate substantial amounts (30-80 μg) of CE (1). Table I lists the fatty acid distribution of CE-fatty acids of control Fu5AH cells grown in MEM supplemented with 10% fetal bovine serum, and of d<1.019 lipoprotein isolated from hyperlipemic rabbit serum. Oleic acid is the predominant fatty acid (73%) in CE from control cells. As shown by others (19), hyperlipemic d<1.019 lipoprotein contains large percentages of both cholesteryl oleate and cholesteryl linoleate. The most marked difference between the cells and the lipoprotein is found in the percentage of linoleic acid (0.8% vs. 31.9%), which serves as a natural marker for lipoprotein CE-fatty acids in this system.

If the major mechanisms of cellular CE accumulation were by the nonselective intracellular accumulation of unhydrolyzed lipoprotein-CE, the fatty acid distribution of the accumulated CE would resemble that of the lipoprotein. As shown in Table I, cells incubated for 24 hr with d<1.019 lipoprotein accumulated CE whose fatty acid distribution did not resemble that of the lipoprotein, containing only 5.6% of linoleic acid in contrast with 31.9% in the lipoprotein.

Incorporation of Exogenous Free Fatty Acids into Cholesteryl Ester

Previous experiments (5) demonstrated that exogenous FC could be taken up by the cells and esterified to form CE. Experiments were now performed to demonstrate that exogenous free fatty acids contribute to the intracellular pool of fatty acids from which esterification with cholesterol occurs. Neither Fu5AH cells, nor hyperlipdemic rabbit d<1.019 lipoprotein, nor cells incubated with this lipoprotein, con-

tain appreciable amounts of linolenic acid (*Table I*). However, cells incubated in medium supplemented with d<1.019 lipoprotein together with linolenic acid bound to albumin accumulated CE containing substantial amounts of linolenic acid, thus demonstrating that exogenous free fatty acids are esterified to cholesterol by the cells.

Incorporation of Endogenous Fatty Acids into Cholesteryl Ester

An experimental system was designed to determine the contribution of endogenous fatty acids to cellular esterification of cholesterol. This was accomplished through the use of AH-DLP cells in which the endogenous (zero time) fatty acid had been prelabeled to a constant specific activity by prolonged growth in medium containing ^{14}C-glucose. From comparisons of the specific activity of intracellularly accumulated CE-fatty acids with the initial specific activity of endogenous cellular fatty acids, the percentage contribution to CE-fatty acids from endogenous fatty acids was calculated (see Methods). The results of a typical experiment are presented in Table II. Both hyperlipemic serum and d<1.019 lipoprotein were used to stimulate cellular CE accumulation. The percentages of CE-fatty acids derived from endogenous fatty acids following exposure to hyperlipemic serum and d<1.019 lipoprotein were 36% and 45%, respectively. Cellular total fatty acid specific activity was also determined and was found to be markedly diluted with exogenous unlabeled fatty acids.

Effect of Chloroquine

It has recently been reported that the presence of chloroquine inhibits cellular cholesteryl ester hydrolase (EC 3.1.1.13) and modifies the fatty acid composition of cellular esterified cholesterol in fibroblast cultures (20). The data presented in Table III demonstrate that, in the Fu5AH system, chloroquine acts as a potent inhibitor of cholesterol esterification. With increasing doses of chloroquine, cellular CE content decreases and cellular FC increases. These changes in cholesterol content can, in part, be explained by the data on cellular FC incorporation (Table III), which demonstrate a continued incorporation of exogenous FC into cellular total cholesterol, accompanied by marked inhibition of the esterification of FC. Thus, the percentage of incorporated FC that was esterified decreased from 66% in control cells to 7% in cells treated with 50 μg/ml chloroquine.

The effect of this inhibitor of cholesterol

TABLE III

Effect of Chloroquine on Cholesterol Metabolism in Fu5AH Cells Grown in Hyperlipemic Serum[a]

Chloroquine (μg/ml)	Cellular cholesterol content (μg/mg protein)[b]			Exogenous FC incorporation[c] (μg/mg protein/24 hr)[b]			
	FC	CE	TC	FC	CE	TC	% Esterification[d]
0	22.0 ± 2.0	73.0 ± 6.0	95.3 ± 6.6	9.6 ± 0.5	19.4 ± 4.1	29.1 ± 4.0	66.4 ± 5.1
25	29.6 ± 1.8	66.5 ± 15.4	96.1 ± 15.9	17.0 ± 1.4	9.3 ± 4.7	26.3 ± 5.7	33.6 ± 10.7
50	40.3 ± 2.7	32.5 ± 14.1	72.7 ± 16.0	22.8 ± 7.0	1.7 ± 0.4	24.5 ± 6.7	7.5 ± 3.1

[a]Cells incubated 24 hours with 2.5% hyperlipemic rabbit serum labeled with 4-[^{14}C]-cholesterol, and either 0, 25, or 50 μg/ml of chloroquine. FC = free cholesterol, CE = cholesteryl esters, TC = total cholesterol.

[b]Mean ± Standard Deviation of four independent determinations.

[c]Calculated from exogenous FC specific activity (See Methods).

[d]% esterification = cpm in CE/ (cpm in FC + cpm in CE).

esterification on the origin of cellular CE fatty acids was studied using labeled AH-DLP cells (Table II). Incubation with d<1.019 lipoprotein alone resulted in accumulation of CE in which 45% of the fatty acids were derived from endogenous fatty acids. When cholesterol esterification was inhibited with chloroquine, only 4% of the CE-fatty acids were derived from endogenous fatty acids.

DISCUSSION

The fatty acid distribution of the CE accumulated by Fu5AH cells incubated with hyperlipemic rabbit d<1.019 lipoprotein (Table I) was marked by a higher percentage of oleic acid and a much lower percentage of linoleic acid than that of the d<1.019 lipoprotein CE to which the cells were exposed. These results are qualitatively similar to those of Smith et al. (21) in studies of human atherosclerotic aorta. They found that intracellular accumulations of lipid contained CE having a lower percentage of linoleic acid and a higher percentage of oleic acid than serum s_f 0-12 lipoprotein. A high percentage of cholesteryl oleate and a low percentage of cholesteryl linoleate have been reported by Lang and Insull (2) in the lipid inclusions of human aortic atheromata. Morphologically similar intracellular inclusions, consisting of both isotropic and anisotropic types, are found in Fu5AH cells after incubation with hyperlipemic serum (4). The major differences between the CE-fatty acids of Fu5AH cells incubated with d<1.019 lipoprotein and those of the lipoprotein itself demonstrate that cellular CE accumulation is not simply the result of nonselective uptake and accumulation of lipoprotein CE. Another mechanism must be involved to account for the fatty acid distribution seen. Available data on lipoprotein structure (22,23) and uptake (24,25) make it unlikely that selective uptake of lipoprotein CE occurs. Selective hydrolysis of incorporated lipoprotein CE has been suggested in studies of cholesteryl ester hydrolase (26-28). Many studies with a variety of cell (5), organ (29,30), and whole animal (31-33) systems have demonstrated a role for cellular esterification of cholesterol.

When linolenic acid, as the free fatty acid bound to albumin, was added to the incubation medium (Table I), substantial amounts of linolenic acid were found esterified to cholesterol. Such incorporation demonstrates that cellular esterification of cholesterol plays a role in cholesteryl ester accumulation. These data are consistent wih earlier studies (5) which showed an active esterification of cholesterol by Fu5AH cells when incubated in the presence of hyperlipemic serum or hyperlipemic lipoproteins, and with those of other investigators working with a variety of experimental systems (29-33).

The isotopic experiments described here were designed to determine the relative contributions of endogenous and exogenous fatty acids to the cholesteryl ester accumulated by cells incubated with hyperlipemic serum. From experiments with AH-DLP cells containing ^{14}C-labeled intracellular fatty acids (Table II), it was calculated that ca. 1/3 of the fatty acids which accumulate as CE in 24 hr were derived from fatty acids present in the cell at the time of addition of hyperlipemic serum. It should be noted that if a substantial amount of fatty acid synthesis were occurring during the time of exposure to hyperlipemic serum, the results would represent the percentage of fatty acid-carbon derived from endogenous fatty acids. The duration (24 hr) of the incubation in our experiments makes it unlikely that a significant amount of fatty acid synthesis occurs. Furthermore, preliminary studies in our laboratory suggest that fatty acid synthesis in AH-DLP cells is inhibited in the presence of hyperlipemic serum.

These data also demonstrate that a substantial amount of cellular CE is a product of cellular esterification. One-third is a minimum estimate of the contribution of cellular esterification to the total cellular accumulation of CE. This represents only the contribution of endogenous fatty acids to the process of esterification and does not include the contributions of fatty acids derived from exogenous lipids. The experiments in which cells were incubated with exogenous linolenic acid demonstrated that exogenous free fatty acids were esterified to form CE (Table I). The marked dilution of cellular total fatty acid specific activity by exogenous lipid (Table II) demonstrates extensive incorporation of exogenous fatty acids. If throughout the accumulation period, the fatty acids becoming esterified to cholesterol had a specific activity similar to the total fatty acid specific activity, then the esterification process would account for 80-90% of the accumulation of CE and would be the primary mechanism for the accumulation of esterified cholesterol.

Chloroquine has been reported to be taken up by cultured fibroblasts and concentrated in lysosomes (34), and to inhibit lysosomal hydrolysis of protein (34) and of CE (20). We have found that it partially inhibits cholesterol esterification in Fu5AH cells (Table III). When cellular esterification is inhibitied by chloroquine, CE content is decreased and the contri-

bution of endogenous fatty acids to cellular CE is markedly reduced (Table II). This further demonstrates that cellular esterification normally, in uninhibited cells, plays a significant role in cellular CE accumulation.

An accumulation of cholesteryl ester by arterial cells occurs during atherogenesis. The mechanism of this accumulation has been the subject of much debate. It has been suggested that the accumulated CE represents unhydrolyzed lipoprotein-CE (35,36). Some studies have demonstrated that at least part of this CE is a product of cellular esterification (32,33). Our data indicate that a major portion of the CE accumulated by Fu5AH cells exposed to hyperlipemic sera is derived from cellular esterification and that the fatty acids used for esterification are derived both from fatty acids present in the cell at the beginning of the accumulation period and from exogenous fatty acids present in the serum.

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Gas Chromatographic Separation of Cholesteryl Esters of Fatty Acids of Different Degrees of Unsaturation

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ABSTRACT

Cholesteryl esters prepared from the fatty acid methyl esters of linseed oil, pig liver lipids, and Japanese anchovy oil have been separated on the basis of their chain lengths and number of double bonds by gas liquid chromatography on a cyanosiloxane SILAR 10C column. The equivalent chain lengths of cholesteryl esters having acyl groups with 14-22 carbons and 0-6 double bonds are presented. A significant influence of the column temperature on the equivalent chain lengths of the polyenoic fatty acid cholesteryl esters has been found. Separation of the cholestanyl and epicholestanyl esters of linseed oil fatty acids, respectively, under the same conditions is also described.

INTRODUCTION

Developments in gas liquid chromatography (GLC) now permit the direct analysis of the fatty acid cholesteryl esters as described in reviews (1,2). The fatty acid cholesteryl esters require columns and operating conditions similar to those employed in GLC of triglycerides and are usually carried out on a thermally stable nonpolar phase such as SE-30 (3), JXR (4), and OV-17 (4). The results have usually been presented in compositions based on the chain length only.

Kuksis presented two methods for the separation of saturated and unsaturated fatty acid cholesteryl esters in his review (1): GLC on a nonpolar column following a preliminary separation of the mixture on the basis of unsaturation by argentation chromatography, and GLC on thin-film packing containing polyester liquid phase. However, the former techniques are not simple and the latter seems to be impractical since thermal decomposition of the polyester takes place at temperatures near 300 C.

Recently, Murata et al. reported the direct analysis of fatty acid cholesteryl esters on the basis of the chain length and number of double bonds with a mass spectrometer and a chemical ionization source (5). The method seems to be rapid and simple. However, the availability of such instruments is limited and some of the pure specimens required for the analysis are not available at the present time.

This paper presents a new GLC technique for the resolution of cholesteryl esters of saturated and unsaturated fatty acids on SILAR 10C, which was formerly APOLAR 10C as described in the previous paper (6). Though the conditions required are more severe than those used for the wax esters (6), reproducible results were obtained without the base line drift, and the liquid phase could be used over a fairly long time.

MATERIALS AND METHODS

The preparation of fatty acid esters from

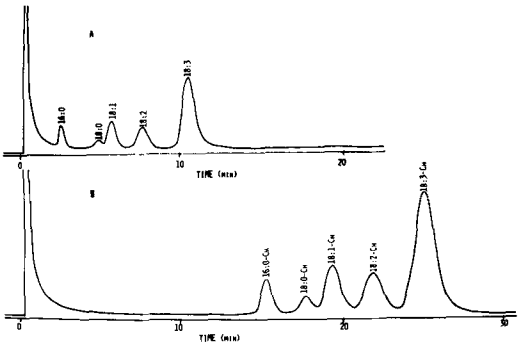

FIG. 1. Gas chromatograms of methyl esters (A) and cholesteryl esters (B) of linseed oil fatty acids. Column; length 0.5 m, A 150 C, B 240-270 C (2 C/min).

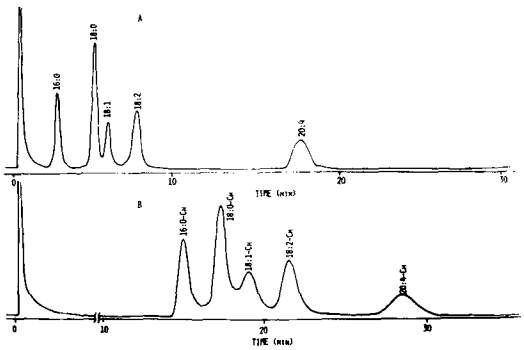

FIG. 2. Gas chromatograms of methyl esters (A) and cholesteryl esters (B) of pig liver lipid fatty acids. Column; length A 1.5 m, B 0.5 m, A 180 C, B 240-270 C (2 C/min).

cholesteryl acetate and linseed oil methyl esters was carried out by a modified transesterification method (7,8). A mixture of linseed oil methyl esters (1.0 g), cholesteryl acetate (1.5 g), dioxane (2 ml), and potassium t-butoxide (50 mg) was heated at 70 C for 1 hr under nitrogen. The cholesteryl esters of the fatty acids from pig liver lipids and Japanese anchovy oil, and the cholestanyl and epicholestanyl esters of the fatty acids from linseed oil were prepared by the same procedure. The wax esters were synthesized as described in the literature (7) by heating of the mixtures of methyl esters (1.0 g) and fatty alcohol (0.5 g) with sodium methylate (40 mg) at 70 C for 40 min. The methyl esters were prepared from linseed oil, Japanese anchovy oil, and erucic acid. Cetyl, stearyl, oleyl, and erucyl alcohols

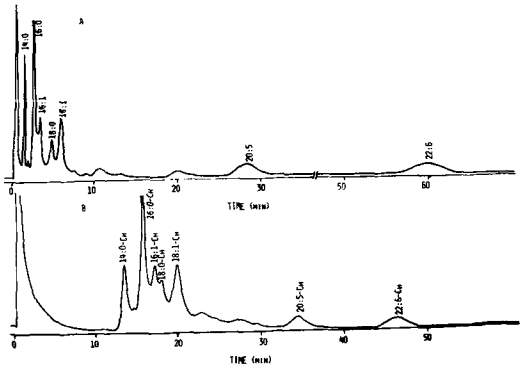

FIG. 3. Gas chromatograms of methyl esters (A) and cholesteryl esters (B) of Japanese anchovy oil fatty acids. Column; length 0.5 m, A 150 C, B 240-270 (2 C/min).

TABLE I

Relative Retention Times and ECL Values of Cholesteryl Esters in Comparison with Those of Methyl and Stearyl Esters[a]

Acyl group	Cholesteryl ester[b]		Stearyl ester[c]		Methyl ester[d]	
	RRT	ECL	RRT	ECL	RRT	ECL
14:0	1.00	---	1.00	---	1.00	---
16:0	1.30	---	1.36	---	1.62	---
16:1	1.56	17.5	1.59	16.9	1.99	16.8
18:0	1.68	---	1.86	---	2.69	---
18:1	1.98	19.3	2.13	18.9	3.24	18.7
18:2	2.44	20.9	2.55	20.0	4.17	19.8
18:3	3.03	22.6	3.11	21.2	5.59	20.9
20:0	2.17	---	---	---	---	---
20:4	3.62	24.0	---	---	---	---
20:5	4.47	25.6	5.12	24.3	12.9	24.4
22:6	6.44	28.5	7.68	26.9	25.0	27.1

[a]ECL = equivalent chain length based on the carbon number of acyl groups, RRT = relative retention time (standard, myristate). Retention times of myristates (min); cholesteryl 5.14, stearyl 3.99, methyl 2.50. Glass columns of inside diameter 3 mm packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh) were used to obtain the data in every case.

[b]Column temperature 270 C. Column length 0.5 m.

[c]Column temperature 250 C. Column length 1.5 m.

[d]Column temperature 180 C. Column length 1.5 m.

TABLE II

Separation Factors of Fatty Acid Esters on SILAR 10C[a]

Molecular species	Cholesteryl ester	Stearyl ester	Methyl ester
Monoene/Saturate[b]	1.19	1.16	1.22
Diene/Monoene	1.23	1.20	1.29
Triene/Diene	1.24	1.22	1.34
C_{n+2}/C_n[c]	1.29	1.36	1.64

[a]Separation factors are average values calculated from relative retention times (RRT) listed in Table I.

[b]Calculated from the RRT of each ester having the acyl groups of same carbon number.

[c]Separation factors of esters having the same number of double bonds; n = carbon number of acyl groups.

TABLE III

Comparison of Δ_t ECL of Cholesteryl and Methyl Esters[a]

Acyl group	Cholesteryl ester[b]			Methyl ester[c]	
	240-255	250-260	255-265	DEGS	SILAR 10C
18:1	0.0040	0.0083	0.0157	0.0040	0.0068
18:2	0.0058	0.0167	0.0272	0.0050	0.0108
18:3	0.0112	0.0263	0.0410	0.0080	0.0135

[a]Δ_t ECL; increase of equivalent chain length (ECL) per one degree rise in column temperature.

[b]240-255 indicates that the Δ_t ECL was calculated from ECL at 240 and 255 C.

[c]The Δ_t ECL was calculated from ECL at 150 and 190 C.

TABLE IV

Compositions of Cholesteryl Esters in Comparison with Those of Original Methyl Esters[a]

Acyl group	Cholesteryl ester	Methyl ester
16:0	5.8	6.0
18:0	2.8	3.5
18:1	16.0	15.3
18:2	15.5	15.7
18:3	59.9	59.5

[a]Percents of peak area.

were used for the esterification.

The GLC of cholesteryl esters was performed with a Shimadzu GC 6AM instrument equipped with dual glass columns (0.5 m length, 3 mm inside diameter) and flame ionization detectors. The columns were packed with Gas Chrom Q (100-120 mesh) coated with 5% SILAR 10C (Applied Science Laboratories, State College, PA). The carrier gas was nitrogen at 40 ml/min. Prior to use, the columns were conditioned at 270 C for 10 hr. Detector and injection heaters were maintained at 320 C. The column temperature was kept at 270 C or programmed from 240-270 C at the rate of 2 C/min from the start of the analysis. Difficulties in determining peak areas due to partial overlapping were overcome by a digital integrator (Shimadzu ITG-4AX). The peaks were identified by comparison of the synthesized cholesteryl esters with appropriate reference standards. After the column was cooled to the initial temperature, sufficient time was taken for the SILAR 10C to revert back to the original polarity. An analogous alteration of the liquid phase polarity, dependent upon temperature, was reported with the cyanosiloxane XE-60 (9).

RESULTS AND DISCUSSION

Figures 1-3 compare the typical chromatograms obtained for the cholesteryl esters prepared from methyl esters of linseed oil, pig liver lipids, and Japanese anchovy oil, with those for the original methyl esters, respectively. After examining columns 0.3-1.5 m in length, a 0.5 m column was selected to finish the total analysis from cholesteryl myristate (14:0-Ch) to cholesteryl docosahexaenoate (22:6-Ch) within 1 hr. For good resolution of the cholesteryl esters of some vegetable oil fatty acids, a 1.5 m column is preferable. The elution patterns are characterized by a fairly good resolution of the various components as shown in Figures 1-3, except for the poor separation of stearate and hexadecenoate peaks in Figure 3. The column temperature used in this study is somewhat low compared with those which have been reported

TABLE V

Comparison of Retention Times and ECL Values of Fatty Acid Epicholestanyl, Cholestanyl, and Cholesteryl Esters

Acyl group	Epicholestanyl ester		Cholestanyl and cholesteryl esters	
	Retention time (min)	ECL[a]	Retention time (min)	ECL[a]
16:0	4.25	—	6.75	—
18:0	5.45	—	8.78	—
18:1	6.27	19.1	10.3	19.3
18:2	7.56	20.6	12.6	20.9
18:3	9.28	22.3	15.5	22.6

[a]Equivalent chain length (ECL) based on the carbon number of acyl groups.

in previous papers (1-4). This is permitted by the low affinity of the SILAR 10C polar liquid phase to the cholesteryl esters which have a low polarity. Table I compares the relative retention times (RRT) with respect to myristate and equivalent chain lengths (ECL) based on the chain lengths of the acyl groups for cholesteryl, stearyl, and methyl esters. Cholesteryl esters of unsaturated fatty acids show more or less higher ECL than the corresponding stearyl and methyl esters while ECL of the latter shows quite similar values. This is to be expected since the slopes of the lines in plotting $\log_{10}$ retention time against chain lengths for wax esters and cholesteryl esters are different as described later. The separation factors calculated for the cholesteryl, stearyl, and methyl esters are summarized in Table II. The separation factors of the cholesteryl esters, based on similar degrees of unsaturation and fatty acid chain lengths, are close to those of wax esters but lower than those of methyl esters. This is evidently due to the decrease of resolution from the higher molecular weights of the cholesteryl esters.

In comparison with SILAR 5CP, SILAR 10C liquid phase showed high separation factors for the double bond homologues and the low separation factors for the chain length homologues (10,11). This characteristic is most significant in cholesteryl esters. In practice, this is recognizable from the fact that linoleate elutes after arachidate in GLC of cholesteryl esters, whereas the former usually emerges before the latter in the methyl esters, and the former overlaps with the latter at 250 C in the wax esters. The influence of the column temperature on the separation patterns of the methyl esters on the polyester columns was reported in detail by Ackman (12).

The wax esters, stearyl behenoate (18:0-22:0), the saturated components of 52 carbons in beeswax and erucyl docosahexaenoate (22:1-22:6) showed the retention times corresponding to ca. 1:0-Ch, 14:0-Ch, and 11:0-Ch in the cholesteryl ester series, respectively (13). Thus, mixtures of wax esters and cholesteryl esters can be separated on SILAR 10C, if the sample does not contain cholesteryl esters shorter than laurate and wax esters longer than 22:1-22:6 on the retention time base. The influence of column temperature on the ECL can be shown by Δ_t ECL, the increase of ECL for one degree rise of column temperature. The Δ_t ECL values of cholesteryl and methyl esters are shown in Table III. It indicates that Δ_t ECL values of cholesteryl esters increase with increasing degree of unsaturation and increase of column temperature. The data demonstrate that the overlapping components having different degrees of unsaturation could be separated by selection of a more appropriate column temperature, and that the ECL of polyenoic acid cholesteryl esters should be reported together with a record of the column temperature.

The facts concerning the influence of column temperature on the separation factors were reported in the GLC of the esters on polar columns in a few papers. In the GLC of methyl esters on DEGS and an organosilicone polyester EGSS, it was found that the ECL values of unsaturated esters change with the temperature since the slopes of the lines due to unsaturated esters relative to the saturated esters alter with temperature when plotting $\log_{10}$ retention time against carbon number (12,14,15). In the GLC of trimethylsilyl ethers of diacylglycerols on SILAR 5CP, the relative temperature dependence of separation by carbon number and degree of unsaturation can be illustrated by plotting the ECL of diacylglycerols versus the sum of the ECL values of methyl esters of the fatty acids (10). Although nearly straight lines are obtained for the diacylglycerols from seed oils, the polyunsaturated species present in phospholipids have been found to deviate from this relationship. It was suggested that the esters containing polyunsaturated species do not possess the same temperature dependence (10).

The results in Table III show that Δ_t ECL of an unsaturated methyl ester on SILAR 10C phase is higher than the Δ_t ECL on DEGS. This suggests that SILAR 10C is more sensitive to temperature effects. This effect can be ascribed to conformational changes of polymers, which have bulky polar side chains, with increases of thermal energy as described in GLC on XE-60 (9).

The peak area compositions of cholesteryl esters of linseed oil fatty acids were determined by GLC on SILAR 10C columns. As shown in Table IV, the proportions of the cholesteryl esters were in fair agreement with those of original methyl esters. Further work, however, is now in progress to demonstrate more reliable results for the quantification of cholesteryl esters on SILAR 10C.

Table V shows the ECL of the cholesteryl esters of linseed oil fatty acids. The ECL values based on the carbon number of the acyl groups are in both cases in agreement with those of the corresponding cholesteryl esters. The steric configuration of the 3-OH has a significant influence on the ECL values, but the absence of 5:6-double bond has little influence in the cholestanyl esters. Thus, the GLC on SILAR 10C is expected to be useful for the characteri-

zation of the fatty acid esters of various sterols.

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The Triterpenes of Ouricuri Wax[1]

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ABSTRACT

Ouricuri wax contains lupeyl acetate (3.9%), taraxerone (1.3%), lupenone (1.2%), simiarenol (0.09%), and taraxeryl acetate (0.03%). The triterpenes can be readily isolated from the wax by hexane extraction. Gas liquid chromatographic analysis shows that lupeyl acetate is the principal single component of the wax.

INTRODUCTION

Ouricuri wax, a hard, dark brown wax sometimes used as a substitute for carnauba wax, is obtained from the leaves of the ouricuri palm, *Syagrus coronata* (1,2). During an investigation of gas liquid chromatographic (GLC) analysis of unhydrolyzed commercial waxes, it was observed that the chromatogram of ouricuri wax contained a prominent single peak close to the emergence position of triacontyl acetate (3). The emergence time was unaffected by acetylation and, on acid methanolysis, the unknown component appeared to be converted to an alcohol (3). The component apparently formed about 5% of the wax and was thus the most abundant single component. A prominent peak, thought to be due to a long chain alcohol, had also been observed earlier in GLC analysis of ouricuri wax but was not identified (4).

It has now been shown that the unknown component is the triterpene lupeyl acetate, and this compound, together with lesser amounts of other triterpenes, forms about 6.5% of ouricuri wax.

EXPERIMENTAL PROCEDURES

GLC analyses were carried out as previously described (5); relative emergence temperatures were: lupeyl acetate 1, taraxeryl acetate 0.97, lupenone 0.96, and taraxerone 0.92.

Chloroform containing 1% v/v EtOH was the solvent for thin layer chromatography (TLC) on Silica Gel G, and compounds were made visible by charring after spraying with 50% H_2SO_4. R_f values were: lupeyl and taraxeryl acetates 0.47, taraxerone and lupenone 0.35, simiarenol 0.23, and lupeol 0.18; and for comparison, the R_f value of triacontanol was 0.20.

Mass spectra (MS) and ^1H nuclear magnetic resonance (PMR) spectra (in $CDCl_3$) were measured as previously described (5).

Powdered ouricuri wax (Dilmont Inc., Montreal) (500 g) was stirred with hexane and the slurry poured into a chromatographic column. Hexane was then allowed to percolate through the column and three 1.5 liter fractions were collected; the first (I) contained 76 g; the second (II), 8 g; and the third (III), 3.5 g. GLC showed that I was mainly lupeyl acetate with lesser amounts of lupenone and taraxerone and that II and III contained some triterpene acetates and taraxerone together with larger amounts of long chain alcohols, acids, and esters. TLC confirmed these identifications.

Fraction I was crystallized from 350 ml hexane at 25 C and gave a solid fraction (IA, 7.1 g) and a more soluble residue (IB, 68.9 g). IA contained a major proportion of taraxerone with some lupeyl acetate, alcohols, and more polar components (according to TLC). Fraction IB, IA, and II and III combined were then fractionated by column chromatography on silicic acid (Biosil A, Biorad Laboratories, Richmond, CA) with hexane containing increasing amounts of ether as solvent.

IB (11.8 g) gave wax hydrocarbons (0.75 g, elution with hexane), long chain esters (1.4 g), lupeyl acetate (3.13 g), and mixtures of taraxerone and lupenone (0.8 g) (all eluted with hexane containing 0.5% ether). Taraxerone was separated from lupenone by fractional crystallization from hexane. Elution with hexane-1% ether gave lupenone (0.5 g) and with hexane-5% ether gave crude simiarenol (0.16 g). Further elution with the latter solvent and with hexane containing up to 25% ether gave mixtures of long chain alcohols and triterpene alcohols (probably lupeol from PMR), long chain acids and a complex mixture apparently containing hydroxy acids and diols (TLC). Chromatography of IA (7.1 g) gave triterpene acetates (1.15 g) which were separated into soluble lupeyl acetate (1 g) and insoluble taraxeryl acetate (0.15 g) by acetone extraction. Later fractions contained taraxerone (2.75 g) and were followed by mixed fractions similar to the later fractions from IB. Chromatography of II and III gave complex mixtures of mainly aliphatic compounds and a very little crude triterpene ketones.

Identification of the triterpenes followed from the physical properties listed below.

Lupeyl acetate, long needles from acetone,

[1]NRCC No. 15667

had m.p. 220-221 C [reported (6) m.p. is 220 C]; PMR: δ 0.79, 0.85-0.87 (9 protons). 0.94, 1.03 (all s, CH_3), 1.67 (s, H-30), 2.02 (s, acetate). 4.47 (m, H-3), 4.54, 4.67 (apparent d, H-29). The MS was very similar to that of an authentic sample.

Lupenone, needles from acetone had m.p. 168-172 C [reported (6) m.p. is 170-171 C]; PMR δ 0.80, 0.93, 0.96, 1.02, 1.07 (6 protons) (all s, CH_3), 1.67 (s, H-30), 4.55, 4.65 (m, H-29); MS m/e (rel. int.): 424 M^+ (53), 409 (11), 314 (14), 313 (18), 245 (18), 218 (20), 205 (66), 189 (31), 55 (100). The MS has been interpreted by Budzikiewicz et al. (7).

Taraxeryl acetate, rods from chloroform, had m.p. 305-307 C [reported (8) m.p. is 298-305 C]; PMR: δ 0.87, 0.90, 0.92, 0.96 (6 protons) 1.01 (6 portons), 1.14 (all s, CH_3), 2.05 (s, acetate), 4.48 (t, H-3), 5.54 (q, H-15); MS m/e (rel. int.): 468 M^+ (12), 453 (5), 344 (35), 329 (11), 269 (8), 218 (11), 204 (100), 135 (35), 133 (29), 121 (23), 119 (23).

Taraxerone, long needles from benzene, had m.p. 245-247 C [reported (8) m.p. was 240-249 C]; PMR: δ 0.83, 0.92 (6 protons), 0.95, 1.05, 1.08 (6 protons), 1.14 (all s, CH_3), 5.54 (q, H-15); MS m/e (rel. int.): 424 M^+ (63), 409 (24), 300 (52), 285 (28), 218 (41), 205 (52), 204 (86), 189 (27), 133 (56), 121 (42), 41 (100).

The principal peaks in the MS of taraxeryl acetate and taraxerone have been interpreted previously (7).

Simiarenol, large plates from hexane, had m.p. 218-220 C [reported (9) m.p. is 210 C]; PMR: δ 0.78, 0.85, 0.88, 0.92, 1.00, 1.03, 1.13 (all s, CH_3), 3.45 (m, line width at half height 7 Hz, H-3), 5.60 (m, H-6); MS m/e (rel. int.): 426 M^+ (26), 411 (4), 408 (3), 274 (100), 259 (52), 245 (7), 231 (13), 205 (11), 175 (9), 152 (40), 134 (54), 122 (36). The PMR spectrum and the MS were very similar to those of an authentic sample supplied by W.W. Hui. The principal peaks of the MS have been interpreted by Aplin et al. (9).

RESULTS AND DISCUSSION

Ouricuri wax contains lupeyl acetate (3.85%), taraxerone (1.33%), lupenone (1.24%), simiarenol (0.09%), and taraxeryl acetate (0.03%). Lupeol is one of the most common triterpenes (6), and the acetate, which has been reported to be a major constituent of leaf wax of two *Acacia* species (10), has been frequently isolated (6). Since ouricuri wax is commerically available, and since the above isolation procedure is relatively simple, it would be a convenient source of the acetate. Lupenone has also been found in nature previously (11). Taraxerone is a fairly common triterpene (8), and taraxeryl acetate has also been isolated before (8). Simiarenol, which is much less common, was previously isolated from leaves of *Rhododendron simiarum* (9).

These triterpenes may not be true constituents of epicuticular wax of the ouricuri palm, but since wax is scraped from the leaves by hand (1,2), they may be derived from the interior of the leaves. However, the total of at least 6.5% of triterpenes may be sufficient to affect the properties of ouricuri wax and the acetates would contribute to the relatively high ester value.

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[Received September 20, 1976]

SHORT COMMUNICATIONS

Absence of Eicosatrienoic Acid in Plasma of Sheep Parenterally Infused with a High Glucose Fat-Free Solution[1]

ABSTRACT

Five yearling wether sheep were maintained on total intravenous, fat-free feeding for periods in excess of 4 weeks. Analysis of plasma total lipid, neutral lipid, and phospholipid fatty acid patterns showed a decrease in linoleic acid during the first week, after which levels tended to stabilize. Rate of decrease in linoleic acid content of plasma phospholipids of two sheep was different. Changes in nonessential fatty acids were variable. 5,8,11- and 8,11,14-Eicosatrienoic acids were not detected in any of the sheep.

INTRODUCTION

Essential fatty acid (EFA) requirements of ruminants are not well-defined. Sklan et al. (1) concluded that essential fatty acid requirements of new-born calves were "very low"; however, Lambert et al. (2) were able to demonstrate retarded growth at 3 wk in calves fed a lipid-free diet, and EFA deficiency symptoms at 6 wk. New-born lambs are EFA deficient, as characterized by a plasma phospholipid triene/tetraene ratio greater than 0.4 (3). The triene/tetraene ratio decreased from 1.0 at birth to 0.08 after 8 days of suckling ewe's milk containing 1-2% linoleic acid in the fat (3). Noble et al. (4) reported that lambs retained all ingested linoleic acid in their tissues up to 20 days of age. Weanling lambs fed a fat-free diet for 7 mo showed no skin lesions or other clinical symptoms of EFA deficiency (5). In light of the apparently high tenacity of ruminant tissues for linoleic acid, we took the opportunity to examine plasma fatty acid composition of yearling wethers maintained on a fat-free intravenous infusion for periods exceeding 4 wk, in conjunction with another experiment (6).

METHODS

Detailed experimental procedures, carried out to examine nitrogen and mineral balance, are reported elsewhere (6). Sheep were fitted with silastic jugular catheters and were kept in individual metabolism crates. They were infused with a balanced electrolyte solution to meet mineral requirements, while energy and nitrogen requirements were provided by infusion of glucose and a commercially-available fibrin hydrolysate ("Aminisol"—modified fibrin hydrolysate, Abbott Laboratories, N. Chicago, IL). Sheep were abruptly changed from oral to intravenous feeding. Nitrogen balance and body weight changes were criteria for adequacy of nitrogen and energy requirements, respectively.

Glucose and amino acid-mineral solutions were kept separate and mixed by the infusion pump immediately prior to infusion. Intra-

[1]Approved as Journal Article No. 102-76, Ohio Agricultural Research and Development Center, Wooster, OH 44691.

TABLE I

Fatty Acid Distribution (Weight % of Total Fatty Acid) in Total Serum Lipids of Three Sheep Parenterally Fed a High Glucose Fat-Free Solution for 4 Weeks. Mean ± Standard Error of Mean

Fatty acid	Week 0	Week 1	Week 2	Week 3	Week 4
14:0	3.8 ± 0.6	8.4 ± 2.1	9.0 ± 2.8	10.1 ± 3.2	7.0 ± 2.1
16:0	24.9 ± 4.7	26.5 ± 3.2	29.2 ± 5.1	32.6 ± 2.6	28.7 ± 4.3
16:1 n-7	5.0 ± 1.5	11.8 ± 3.5	9.4 ± 2.2	12.4 ± 5.6	10.8 ± 4.6
18:0	21.6 ± 3.2	17.7 ± 0.7	15.5 ± 1.8	13.1 ± 4.6	14.7 ± 2.5
18:1 n-9	20.9 ± 0.8	22.9 ± 3.5	22.3 ± 1.2	19.7 ± 7.3	24.0 ± 4.7
18:2 n-6	17.8 ± 3.7	8.0 ± 1.7	7.1 ± 2.1	4.2 ± 1.5	7.4 ± 1.9
18:3 n-3	2.1 ± 0.7	0.4 ± 0.6	0.3 ± 0.1	ND	2.8 ± 1.6
20:3 n-9	ND[a]	ND	ND	ND	ND
20:4 n-6	3.2 ± 0.6	1.8 ± 0.3	3.8 ± 1.0	3.6 ± 0.1	5.4 ± 1.1

[a]ND = not detected.

TABLE II

Fatty Acid Distribution (Weight % of Total Fatty Acid) in Plasma Neutral Lipids of Sheep Parenterally Fed for 4 Weeks

Fatty acid	Week 0	Week 1	Week 2	Week 3	Week 4
			Sheep 4		
14:0	1.9	6.6	6.1	4.6	7.9
16:0	22.2	30.3	36.7	28.1	24.6
16:1 n-7	2.7	8.6	12.3	13.5	8.1
18:0	8.7	12.0	14.7	18.3	15.1
18:1 n-9	26.7	22.9	21.3	24.0	27.2
18:2 n-6	28.8	9.8	4.9	7.6	6.9
18:3 n-3	2.2	1.2	1.1	1.8	0.8
20:3 n-9	ND[a]	ND	ND	ND	ND
20:4 n-6	3.9	4.6	0.4	1.5	2.4
			Sheep 5		
14:0	1.3	1.3	2.8	2.4	6.0
16:0	19.8	35.0	27.7	29.6	33.7
16:1 n-7	4.0	3.6	8.0	10.4	6.9
18:0	12.1	13.5	10.1	6.9	12.8
18:1 n-9	30.7	28.8	28.4	31.3	29.8
18:2 n-6	22.5	7.9	6.9	7.6	4.7
18:3 n-3	2.4	1.3	0.8	0.1	0.2
20:3 n-9	ND	ND	ND	ND	ND
20:4 n-6	2.6	3.3	5.4	6.1	2.0

[a]ND = not detected.

muscular injection of B-vitamins were given daily with the fat-soluble vitamins at 30 day intervals.

Heparinized blood samples collected weekly were extracted twice with 10 volumes of $CHCl_3$:MeOH 2:1. The solvent was removed on a rotary evaporator at 50 C; then samples were directly methylated or dissolved in $CHCl_3$ and applied to a silicic acid column (30 mg lipid/g silicic acid). Neutral lipids were eluted with $CHCl_3$ and polar lipids with methanol. Solvent was removed and the lipids were dissolved in 2 ml benzene. Twenty ml 5% HCl in methanol were added and refluxed for 2 hr. Methyl esters were extracted with a petroleum ether:H_2O system.

Methyl esters of the fatty acids were analyzed on a 4 mm ID x 120 cm glass column packed with 15% HiEff 2BP on 60/80 mesh Gas Chrom P (Applied Science Labs, State College, PA) in an F & M 402 gas chromatograph. Detector output was recorded and electronically integrated with a Hewlett-Packard Model 3380-A programmable digital integrator. Peaks were identified by comparison with authentic standards (Nu-Chek Prep, Elysian, MN). Standards of 5,8,11- and 8,11,14-eicosatrienoic acids were a gift from Dr. Howard Sprecher, Department of Physiological Chemistry, The Ohio State University. Coefficients of variation on replicate samples were 18 and 39% for fatty acids comprising greater and less than 10% of the total area, respectively.

RESULTS AND DISCUSSION

Fatty acid composition of the total serum lipids of three sheep during a 4 wk infusion are shown in Table I. After 1 wk, myristic (14:0) and palmitoleic (16:1 n-7) acids doubled. Stearic acid (18:0) decreased by one-third, and linoleic acid (18:2 n-6) decreased by one-half. Other fatty acids were variable, with no consistent trends. Eicosatrienoic acid (20:3 n-9) was not found.

Fatty acid composition changes in plasma of two individual sheep are shown in Table II for neutral lipids and in Table III for phospholipids. Taken together, both tables show several pertinent points. First, linoleic (18:2) and linolenic (18:3) acid decreased rapidly from control (week 0) levels; whereas, arachidonic (20:4) was variable but seemed to increase in later weeks. Second, differences between the two sheep in retention of 18:2 in phospholipids is apparent. Third, nonessential fatty acids were variable, without showing any definite patterns with time, with the possible exception of palmitoleic (16:1), which tended to increase in the neutral lipid fraction. Fourth, eicosatrienoic acid (20:3 n-9) was not detected in any of the plasma samples.

The inability to detect 5,8,11-eicosatrienoic acid during 4 wk of constantly infusing a glucose energy source contrasts with a recent report in which biochemical signs (increased triene/tetraene ratio) of EFA deficiency were

TABLE III

Fatty Acid Distribution (Weight % of Total Fatty Acid) in Plasma Phospholipids of Sheep Parenterally Fed for 4 Weeks

Fatty acid	Week 0	Week 1	Week 2	Week 3	Week 4
			Sheep 4		
14:0	0.8	3.9	6.6	3.6	4.1
16:0	24.2	28.8	32.9	27.7	26.0
16:1 n-7	1.9	9.9	2.4	1.5	2.3
18:0	30.4	26.3	22.4	22.9	18.6
18:1 n-9	14.3	13.7	19.4	28.1	23.0
18:2 n-6	16.0	6.2	3.2	5.9	3.2
18:3 n-3	0.9	0.1	0.0	2.0	0.1
20:3 n-9	ND[a]	ND	ND	ND	ND
20:4 n-6	8.7	4.7	5.1	4.2	5.1
			Sheep 5		
14:0	3.9	2.9	3.1	2.3	1.7
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16:1 n-7	1.4	1.8	3.7	2.5	1.4
18:0	25.8	28.8	24.7	24.7	28.3
18:1 n-9	25.8	25.9	27.6	25.0	28.6
18:2 n-6	12.8	10.7	9.1	6.6	9.1
18:3 n-3	1.7	1.8	1.6	0.9	1.6
20:3 n-9	ND	ND	ND	ND	ND
20:4 n-6	0.4	1.7	2.8	5.2	8.5

[a]ND = not detected.

reported in humans within 24 hr of initiating a glucose infusion (7), and within a few days in intravenously-fed infants (8). Homeostatic mechanisms were apparently functioning to maintain plasma fatty acid composition after a prolonged lack of dietary EFA. Maximum changes in fatty acid composition occurred at 1 wk. With exception of sheep 4 phospholipids, after 1 wk, 18:2 tended to stabilize in the range of 6-9%, similar to values we have observed in new-born ruminants (unpublished observations). Maintenance of plasma minimal EFA levels may reflect an adaptation of adipose tissue to the high glucose levels, permitting stored linoleic and arachidonic acids to be mobilized, or EFA release from tissue degeneration. Although tissue degeneration was not generally apparent, by weeks 3-4 the bone marrow of these sheep become completely infiltrated with adipose cells (aplastic anemia); however, we have no data to suggest that this was due to EFA deficiency. An alternate explanation is that under conditions of EFA deficiency, turnover of linoleic and arachidonic acids in ruminants becomes very low. Our observation of increasing 20:4 is consistent with a recent report by Galli and Spagnuolo (9), who showed reciprocal changes in 18:2 and 20:4 of milk lipids as dietary 18:2 varied, i.e., 20:4 was highest on low 18:2 diets.

This study, conducted under extreme nutritional conditions of EFA deficiency, confirms the reports of others (1,4), that ruminants (at least wether sheep) are resistant to EFA deficiency. The failure to detect 5,8,11-eicosatrienoic acid (20:3) in the phospholipid fraction suggests a possible lack of enzymes for its synthesis in ruminants. However, this explanation seems unlikely, as 20:3 has been detected in significant quantities in new-born lambs in other laboratories (3,10), as well as our own.

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Glycerol Ester Hydrolase Activity in the Pulp of Unerupted Calves' Teeth[1]

ABSTRACT

The pulp from unerupted calves' teeth was found to contain low levels of glycerol ester hydrolase activity. Solutions in Tris buffer cleared tributyrylglycerol agar and hydrolyzed emulsified olive oil. The average quantity of free fatty acids released in five assays with olive oil was: 2.8, S.D. ± 1.4. Specific activities were: 0.25, S.D. ± 0.13, mkatals/kg protein.

The endogenous pulp of enamel matrix contains tri-(TG), di-(DG), monoacylglycerols (MG) and free fatty acids (FFA) (1-3). The DG, MG, and FFA could be the products of lipase action on the TG, although Rabinowitz et al. (2) attributed their presence to the heat generated during grinding done to remove the enamel in their sample preparation. However, we have obtained positive evidence for lipolytic activity in the pulp, which to our knowledge, has not been reported previously.

In a preliminary screening, portions of the pulp from the unerupted back teeth of a young calf were smeared on tributyrylglycerol agar, and clearing was observed after 3 days at 37 C, which was taken as an indication of lipolytic activity.

The lower jaws from three calves, aged 0-2 wk, were obtained from a packing house and the unerupted teeth freed from the back portion of the jaws with the aid of a Stryker autopsy saw. The yellow gelatinous pulp, removed from the enamel matrix of 10 unerupted teeth with forceps, was pooled and homogenized with Tris buffer (pH 8.0, 0.25 M) in a Waring Blendor.

Three crude extracts were prepared from the pulp of the 10 teeth as follows: (a) 90 g of pulp exogenous to the enamel matrix, in 50 ml of Tris buffer (above); (b) 4.8 g of pulp endogenous to the enamel matrix, in 40 ml Tris buffer; and (c) extract (b) filtered to remove heavy particles and diluted to 50 ml with Tris buffer. An initial screening for lipolytic activity was done on the three extracts using Petri dishes filled with tributyrylglycerol agar containing Thimerosal (4). Aliquots (0.1 ml) of each of the extracts were pipetted into wells made in the chilled tributyrylglycerol agar prior to incubation for 48 hr at 36-42 C (5). Bacterial growth was not seen on the agar. Since the hydrolysis of tributyrylglycerol is only tentative evidence for the presence of lipases, olive oil was used as the substrate for confirming the presence of lipolytic activity.

For each assay, 150 mg of olive oil, purified on neutral alumina (6), was emulsified by sonification into 8 ml of Tris buffer containing 1% gum arabic and 0.5 ml of 0.1 M $CaCl_2$. One ml of extract (c) was added and the reaction allowed to proceed with shaking for 90 min at 37 C. The reaction was terminated with 1 ml of 20% H_2SO_4. Two controls were run with each of the three replicate sets of assays: one without substrate and the other without enzyme extract. In the first two sets, the lipids were extracted from each of the digestion mixtures (7), and the extract titrated with 0.013N alcoholic KOH to the thymol blue endpoint (8). N_2 was bubbled through the solution during the titration to stabilize the endpoint (9).

For a third set of assays, the lipids were extracted and the digestion products were separated by thin layer chromatography (TLC). The plates were developed with a hexane-diethyl ether-acetic acid solvent system (90:30:1) and the lipids made visible with bromthymol blue.

The protein content of extract (c) was determined by the biuret method (10) at 540 mn

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The units of activity, mkatals per kg protein, are typically low and representative of crude tissue homogenates. For example, the specific activity obtained by Assman et al. (12) on crude liver plasma membrane TG lipase can be calculated as 0.051 mkat/kg protein.

Glycerol ester hydrolase (EC 3.1.1.3) activity is present in the endogenous pulp of the enamel matrix of the unerupted calves' teeth, but the role of the enzyme in the metabolism of dental tissue is not known.

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Thin Layer Chromatographic Procedure for Class Separation of Plant Neutral Lipids

ABSTRACT

A thin layer chromatographic method has been developed for the class separation of plant neutral lipids. Utilizing a two-step development in one dimension, lipid mixtures are separated into hydrocarbon waxes, steryl esters, methyl esters, triglycerides, fatty acids, diglycerides, sterols, and monoglycerides. The method may be employed for either qualitative or preparative purposes.

INTRODUCTION

A number of methods are available for the separation of lipids by thin layer chromatography (TLC) (1). Most of them were developed for use with animal and/or polar lipids and are not particularly applicable to the neutral lipids extracted from plant leaf material by organic solvents. For these lipids, a method is required that would separate hydrocarbon waxes, sterols, steryl esters, fatty acids and methyl esters as well as mono-, di-, and triglycerides on both qualitative and preparative scales. Our investigation of reported procedures and possible solvent systems failed to yield one with this capability. Thus, available one-step systems (2,3) did not sharply separate the classes for preparative work, and various two-step systems (4-7) did not resolve the hydrocarbons from the mixture nor clearly separate the more polar

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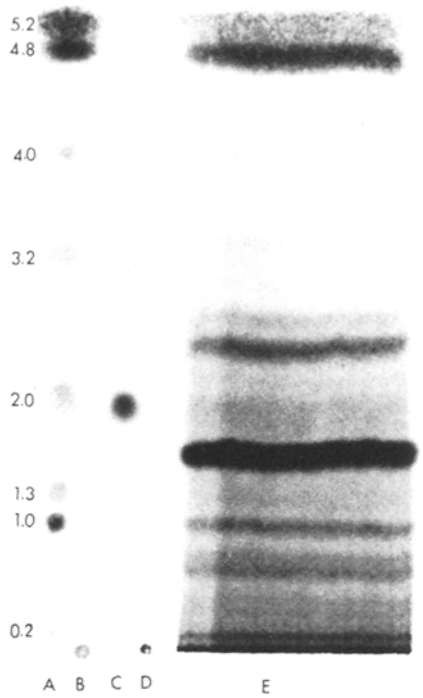

FIG. 1. Thin Layer Chromatogram. A = neutral lipid mixture containing standards identified by R_f's relative to cholesterol (1.0) (5.2 = docosane, 4.8 = cholesteryl oleate, 4.0 = methyl palmitate, 3.2 = glycerol tripalmitate, 2.0 = tetradecanoic acid, 1.3 = glycerol 1,3-dipalmitate, 1.0 = cholesterol, 0.2 = glycerol 1-palmitate); B = chlorophyll mixture; C = xanthophyll; D = β-carotene; E = tobacco extract.

neutral lipids. The use of long (40 cm) TLC plates (8) was inconvenient in terms of amount of solvents and time involved and did not significantly improve resolution.

After experimentation with a number of solvent systems and operating parameters, the method described below was evolved. It may be used for qualitative or preparative TLC and consists of a two-step development in one dimension, with a less polar solvent system first and then with a more polar one. It avoids the use of such toxic or potentially dangerous solvents as benzene and diisopropyl ether.

MATERIALS

All chromatographic solvents were of "distilled-in-glass" grade and were redistilled before use. Lipid standards were docosane and glycerol 1,3-dipalmitate (Analabs, Inc. North Haven, CT); cholesteryl oleate, glycerol 1-palmitate and cholesterol (Applied Science Labs, Inc., State College, PA); methyl palmitate and tetradecanoic acid (ICN-Nutritional Biochemical Co., Cleveland, OH); and glycerol tripalmitate (Sigma Chemical Co., St. Louis, MO). For the purpose of locating common plant materials, other standards used were chlorophyll mixture (Matheson, Coleman & Bell, Norwood, OH); xanthophyll (Pfaltz and Bauer, Inc., Flushing, NY); and β-carotene (ICN-K&K Laboratories, Plainview, NY). Silica Gel G and H were Em Reagents from Brinkmann Instruments, Inc., Westbury, NY.

For qualitative analysis, 20 x 20 cm glass plates were coated according to Stahl (9) with Silica Gel G to a thickness of 250 μ using a Desaga adjustable spreader. For preparative plates, Silica Gel H at a thickness of 2000 μ was used. We chose the latter gel to avoid carryover of binder during elution of components from the plate. The coated plates were air dried for a minimum of 4 hr, then dried at 100 C for 2 hr. They were then washed overnight by ascension of a solution of chloroform:methanol 1:1 v/v, activated at 100 C for 30 min and stored over $CaSO_4$ in a dessicator.

METHODS

For qualitative TLC, lipid samples (2-20 μg/ca. 5 μl hexane) were spotted with an open-end capillary tube at 3 cm from the bottom edge of the plate. On a preparative plate, 1-3 ml of lipid mixture (ca. 25 mg/ml hexane) was streaked across the plate at 3 cm with a sample streaker (Applied Science Labs). When unknown mixtures were chromatographed, lipid standards were spotted or streaked adjacent to the sample.

Qualitative and preparative plates were developed in the same manner. The plate was placed in a lined (Whatman 3 MM paper) chromatography tank containing hexane: diethyl ether 98:2 v/v. Development was allowed to proceed until the solvent front reached the top edge of the plate (20 cm) and then continued for an additional 10 min. After air drying, the plate was redeveloped in a second tank containing hexane:diethyl ether: acetic acid 50:50:1 v/v. When the solvent front reached the upper edge, the plate was removed and air dried. For detection of spots, qualitative plates were sprayed with a solution of sulfuric acid-potassium dichromate (10) and charred at 120 C. In contrast, preparative plates were treated with Rhodamine 6G for visualization in the UV, bands scraped off and components eluted, according to Skipski et al. (11).

RESULTS AND DISCUSSION

Figure 1 illustrates the separation of eight

neutral lipids, each representing a particular lipid class, on a TLC plate of Silica Gel G at 250 μ, by this two-step procedure. All eight classes were well resolved. As an example of the application of this method, the separation of neutral lipids extracted from tobacco leaves is shown on the same plate. Comparisons with reference standards indicated that this sample contained hydrocarbon waxes, steryl esters, fatty acids, and sterols, as well as other unidentified neutral components. Elution of bands from a preparative plate and subsequent analysis by gas chromatography have proven these identifications to be true (J.J. Ellington, P.F. Schlotzhauer, and A.I. Schepartz, unpublished results). For informational purposes the chromatographic response of a chlorophyll mixture, xanthophyll, and β-carotene are also shown. It may be noted that chlorophyll, β-carotene, and the more polar lipids remained at the origin.

Preparative plates with Silica Gel H gave similar results, except that the triglycerides and methyl esters generally ran together. Separations were otherwise good and recoveries were quantitative. Gravimetric recoveries of a separation of the standard mixture of eight neutral lipids (47 mg total in 2 ml hexane) on a preparative plate were between 97 and 103%.

Initially, we investigated the use of commercially prepared silica gel plates from a variety of sources and encountered problems with impurities and reproducibility. Hence, we elected to prepare our own plates. The use of 2000 μ layers for preparative work proved most convenient, enabling the application of samples as large as 80 mg. This may not be the upper limit, since, even at this level, there was no overloading.

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Preparation of Fucosterol from Giant Kelp

ABSTRACT

Commercial extraction of alginic acid from *Macrocystis pyrifera* leaves a residue that is a good source of fucosterol, 5,24(28)E-stigmastadien-3β-ol. The isolation and purification of this sterol is described.

INTRODUCTION

Fucosterol is the predominant sterol in brown algae (1). The giant kelp, *Macrocystis pyrifera*, is a member of this class that grows off the coast of southern California and is used for the preparation of alginic acid in the United States. Attempts are now being made to rear the plant in large marine farms (2). Dried kelp and two by-products of the commercial process, rotary filter mud and kelp slime, were obtained from the Kelco Co., San Diego, CA, for evaluation as easily accessible sources of fucosterol.

EXPERIMENTAL SECTION

Coarsely powdered dry kelp (2 kg) was extracted at room temperature three times with 4 liters 2:1 chloroform:methanol. Evaporation of the combined extracts left 82 g of a dark green residue that was hydrolyzed on the steam bath with 1.2 liters 10% KOH in 95% ethanol for 4 hr. After cooling, the solution was added to 2 liters water and ether extracted to remove

neutral lipids, each representing a particular lipid class, on a TLC plate of Silica Gel G at 250 μ, by this two-step procedure. All eight classes were well resolved. As an example of the application of this method, the separation of neutral lipids extracted from tobacco leaves is shown on the same plate. Comparisons with reference standards indicated that this sample contained hydrocarbon waxes, steryl esters, fatty acids, and sterols, as well as other unidentified neutral components. Elution of bands from a preparative plate and subsequent analysis by gas chromatography have proven these identifications to be true (J.J. Ellington, P.F. Schlotzhauer, and A.I. Schepartz, unpublished results). For informational purposes the chromatographic response of a chlorophyll mixture, xanthophyll, and β-carotene are also shown. It may be noted that chlorophyll, β-carotene, and the more polar lipids remained at the origin.

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7.5 g of a nonsaponifiable fraction which contained 2.3 g fucosterol (0.11% of kelp, estimated by comparison to a standard solution of sitosterol by gas liquid chromatography (GLC): 5% OV-101, 260 C).

Two kg of dry rotary filter mud, the residue left after extraction of alginic acid from the kelp with sodium carbonate solution, was extracted with chloroform-methanol as above to give 32 g of material that was hydrolyzed (500 ml 10% KOH in 95% ethanol, steam bath 4 hr) to yield 8.5 g of a nonsaponifiable fraction containing 3 g fucosterol (0.15% of dry mud).

Four liters kelp slime, the aqueous residue left after alginic acid is precipitated from the carbonate extract with acid, was acidified with 175 ml 37% HCl, heated on the steam bath 3 days to hydrolyze possible steryl glycosides, cooled, and ether extracted. Evaporation of the ether left 2.6 g of a dark tar that contained no fucosterol.

The best source of fucosterol is, therefore, the dry rotary filter mud. It not only contains more sterol than the whole kelp (0.15 vs. 0.11%), but it also contains less chlorophyll and other lipids, making workup easier. The nonsaponifiable fractions from the kelp and mud were combined (16 g), dissolved in benzene (60 ml), and chromatographed on a 200 g column of neutral alumina with the same solvent. Fractions rich in sterol [0.6 to 2.5 liters of eluate, thin layer chromatography (TLC) 60:40 cyclohexane-ethyl acetate] were pooled, concentrated to dryness, and the residue rechromatographed on 200 g of fresh alumina to yield 4.1 g crude fucosterol after crystallization from acetone, m.p. 124-5 C, contaminated with about 5% 24-methylene-cholesterol and a trace of cholesterol (GLC).

The crude material (3.5 g) was acetylated with 10 ml acetic anhydride on the steam bath overnight, dissolved in benzene (20 ml), and chromatographed on a 20% silver nitrate-silica gel column (500 g) with 10:1 hexane-benzene. Fractions were monitored by TLC (10% $AgNO_3$-silica gel plates, 50:50:1 chloroform-carbon tetrachloride-acetic acid); those containing only fucosteryl acetate were combined and the product crystallized from methanol-benzene to yield pure (GLC, TLC) fucosteryl acetate (1.2 g), m.p. 119-20 C (in vacuo, corrected), $[\alpha]_D$ -44.7 C (c5,$CHCl_3$); lit[3] m.p. 120-2 C, $[\alpha]_D$ -42.1 C. The mother liquors from the crystallization of the acetate were evaporated to dryness and the residue hydrolyzed with 10% KOH in 95% ethanol (20 ml) on the steam bath 2 hr. Addition of water to the solution formed a precipitate which was recrystallized from methanol-benzene to yield 0.8 g fucosterol m.p. 123.5-4 C (in vacuo, corrected), $[\alpha]_D$ -42.0 C (c5,$CHCl_3$); lit[3] m.p. 123-4 C, $[\alpha]_D$ -38.5 C.

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A Simple Method for the Preparation of Cholesteryl Esters

ABSTRACT

A simple and convenient procedure for the synthesis of cholesterol esters of long chain saturated and unsaturated fatty acids is presented. Condensation is achieved with thionyl chloride as a catalyst.

INTRODUCTION

The method of using thionyl chloride as a catalyst in the esterification of amino acids (1) has been applied to the synthesis of long chain fatty acid esters of cholesterol. The synthesis was carried out by reacting cholesterol and fatty acid in benzene in the presence of thionyl

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TABLE I

Melting Points and Percentage Yields of Cholesteryl Esters

Product	Melting points (C)		Percentage yields	
	Observed	Reported (5)	At room temperature	At 70 C
Cholesterol oleate	42	42.5	91[a]	96.3[a]
Cholesterol laurate	91	92	90	–
Cholesterol stearate	82	82	90	95.6

[a]The percentage yield represents cholesteryl esters of oleic and linoleic acids.

chloride. Although this reaction proceeded smoothly overnight at room temperature and resulted in high yields (90%), the reaction rate could be increased at higher temperature (70 C), which would result in an overall increase in percentage conversion (96% in 1½ hr). The present method serves as an efficient alternative to other reported methods (2,3).

MATERIALS AND METHODS

A mixture of oleic and linoleic acids and lauric acid, isolated from vegetable fats, were used. Stearic acid and cholesterol of high purity were purchased commercially. Silicic acid (column chromatography grade) was also purchased commercially.

Cholesterol (1.3 mmol, i.e. 500 mg) and a mixture of oleic and linoleic acids (1.6 mmol, i.e. 450 mg) were dissolved in dry benzene and cooled to 0 C. Thionyl chloride (0.25 ml) was added dropwise, and after the flask was flushed with dry carbon dioxide, the reaction mixture was kept overnight at room temperature (25-27 C). Alternatively, the reaction mixture was refluxed at around 72 ± 2 C for 1½ hr. The mixture was transferred to a separating funnel and washed twice with 50 ml of distilled water. The benzene layer was dried over anhydrous sodium sulfate.

The percent conversion of cholesterol to cholesterol ester was determined by thin layer chromatography (TLC). The analytical separation of unreacted cholesterol and free fatty acid from cholesterol ester was achieved in a TLC system Silica Gel G/petroleum ether:diethyl ether:acetic acid 90:10:1 v/v. The separated cholesterol and cholesteryl esters were estimated by the method of Zlatkis et al. (4).

After a mixture of unsaturated cholesteryl esters was isolated in the above system, it was subfractionated by preparative argentation TLC in the system 5% $AgNO_3$ in Silica Gel G/petroleum ether (40-60 C): diethyl ether 90:10 v/v. The separated esters were extracted with 25 ml of chloroform:methanol 1:2, for further characterization.

Cholesterol laurate and stearate were prepared and purified individually by the same procedure.

For the isolation of cholesteryl esters on a macroscale, a silicic acid column 30 cm x 2 cm was used. Silicic acid (80-120 mesh), 50 g was activated overnight at 110 C. Cholesteryl ester was eluted from the column with 1% benzene in petroleum ether (40-60 C). In 75 ml of eluent, the cholesterol ester of a saturated fatty acid was completely removed from the column, recovery (85 ± 2%) being determined either by weighing or by colorimetry. Most of the ester was eluted in the first 25 ml of eluent.

RESULTS AND DISCUSSION

The infrared spectrum of individual cholesteryl esters showed typical strong ester absorption at 1735 cm^{-1}, as reported previously (5). The melting points of cholesteryl oleate, laurate, and stearate resembled closely the values reported in the literature (Table I).

The present method gives yields similar to those reported by Phillips and Viswanathan (2) and Lentz et al. (3) and thus serves as a very useful alternative method in the preparation of cholesteryl esters. When compared to the method of Lentz et al., the present method is a one-step synthesis and hence, should be a preferred choice. The utilization of this method in the synthesis of wax-ester and mono- and diesters of diols and fatty acid derivatives of glycerol will be reported shortly in a separate communication.

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Lipids of Cultured Hepatoma Cells: VIII. Utilization of D-[1-^{14}C] Glucose for Lipid Biosynthesis

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ABSTRACT

Minimal deviation hepatoma 7288C cells (HTC) were incubated in serum-supplemented and serum-free Swim's 77 medium in the presence of D-[1-^{14}C] glucose for 1, 2, 4, 8, 12 and 24 hr. Glucose oxidation to CO_2, incorporation into total cell mass, and incorporation into cell and medium lipids were determined. The percentage distribution of total cell lipid radioactivity in individual neutral and polar lipid classes was followed as a function of time. Degradation studies of individual lipid classes were performed to ascertain the percentage of radioactivity in acyl and glycerol moieties. The percentage of D-[1-^{14}C] glucose oxidized to $^{14}CO_2$, incorporated into cell matter and cell lipids was elevated in cells incubated in serum-free medium as opposed to serum-supplemented medium. The percentage distribution of total cell lipid radioactivity into individual neutral lipid classes from both serum-free and serum-supplemented cultures was as follows: sterols > triglycerides > free fatty acids > sterol esters. The percentage distribution of total cell lipid radioactivity into individual polar lipid classes of serum-supplemented cultures was as follows: phosphatidylcholine > phosphatidylinositol > sphingomyelin > phosphatidylethanolamine > phosphatidylserine. The distribution of glucose radiolabel into individual polar lipid classes of serum-free HTC cells was different from their serum-supplemented counterparts: sphingomyelin > phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine > phosphatidylserine. Glycerol from glyceride classes contained a higher percentage of radioactivity than the acyl moieties, with this percentage significantly elevated in serum-free cultures. The data indicate that, although glucose is a substrate for HTC cell lipids, other precursors present in the culture system also contribute to the lipid constituency of this hepatoma cell line.

INTRODUCTION

Lipids are essential metabolic and structural components of cells (1-5). The numerous observed differences in lipids between normal and neoplastic tissue (6) may play an important role in neoplasia. Cultured Morris minimal deviation hepatoma 7288C cells, called HTC cells (7), are a line of neoplastic cells which are amenable to lipid metabolism studies. Recent work (8) has demonstrated that growth media from HTC cells grown in Swim's 77 medium actually contained more lipids after growth than initially. In addition, lipid classes and fatty acid compositions of HTC cells were influenced only slightly by the amount and composition of serum lipids (9,10). In a recent study (11), it was demonstrated that HTC cells have the capability to carry out de novo fatty acid biosynthesis from ^{14}C acetate. These studies suggested that HTC cells possess the ability to synthesize significant amounts of their cellular lipids, using serum lipids sparingly, conceivably as a source of essential fatty acids. These findings distinguish HTC cells from various other cultured cells (12-17), which significantly utilize exogenous serum lipids. Because exogenous serum lipids do not appear to be a major quantitative contributor of HTC cell lipids, it was reasoned that HTC cells might synthesize most of their lipids de novo from glucose, since glucose is the major component of Swim's 77 medium (18) used to culture HTC cells. This study describes the utilization of glucose by HTC cells for the biosynthesis of cellular lipids.

EXPERIMENTAL PROCEDURE

Media and Growth Conditions

HTC cells were plated out (2 x 10^6 cells/culture flask) and cultured in 75 cm^2 screw cap tissue culture flasks as monolayers in Swim's 77 medium supplemented with 20% bovine serum and 5% fetal calf serum using conventional sterile techniques described previously (8). After the cells had reached confluency (ca. 15 x 10^6 cells/flask), the growth medium was

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decanted and the attached cells rinsed with 0.9% saline. Serum-free Swim's 77 medium without glucose was added to one-half of the flasks (20 ml/flask), while Swim's 77 medium without glucose but containing 5% bovine and 5% fetal calf serum was added to the remaining flasks (20 ml/flask). Serum glucose levels were measured by the glucose oxidase technique (19). D-[1-^{14}C] glucose (1.8 μC/flask) was added to both incubation media. Duplicate flasks for both incubation media were then incubated for 1, 2, 4, 8, 12, and 24 hr. Collection of radioactive CO_2 evolved by the cells was achieved by connecting a section of silicon tubing from the mouth of each culture flask to a scintillation vial containing 250 μl of ethanolamine. After each incubation period, the culture media from the appropriate flasks were collected and the cells harvested by enzymatic release. The collected media were subjected to centrifugation for 160,000 g-min to sediment any particulate matter suspended in the medium. Pelleted material so obtained was combined with the appropriate cell samples. An aliquot of each medium sample was taken to measure ^{14}C radioactivity.

Total Cellular Radioactivity

Two aliquots were taken from each cell sample: one for determination of cellular protein by the method of Lowry (20), and a second for digestion of cell matter with Soluene 350 and subsequent determination of ^{14}C incorporation.

Lipid Extraction and Fractionation

The cell samples were lyophilized, total lipids extracted twice via the Bligh and Dyer procedure (21), and aliquots taken for determination of ^{14}C incorporation. Total lipid extracts were separated into neutral and polar lipid fractions by silicic acid chromatography (22), and the radioactivity in each fraction determined. Individual lipid classes from the neutral lipid and polar lipid fractions were resolved by thin layer chromatography (TLC) as described previously (8), and the amount of ^{14}C incorporation into individual lipid classes measured. Isolated glyceride fractions from a second series of aliquots were saponified by the method of Watson (23) and ^{14}C localization in fatty acid and glycerol moieties of these molecules determined.

Aliquots from the 2 hr and 12 hr incubation media were treated with trichloroacetic acid (TCA) at a final concentration of 10%. Aliquots of the TCA supernatants were spotted on DEAE-cellulose paper and developed by ascending chromatography (24). The DEAE-cellulose strips were cut into segments and the radioactivity determined. TCA supernatants were then extracted twice using the Bligh and Dyer procedure and aliquots of the lipid extracts taken for measurement of ^{14}C radioactivity. TCA precipitates were dissolved in Soluene 350 and aliquots taken for determination of ^{14}C incorporation.

Materials

Plastic tissue culture flasks were purchased from Falcon Plastics, Oxnard, CA. Fetal calf and bovine serum were purchased from Pel-Freez Biologicals, Inc., Rogers, AR, and purified and sterilized via Millipore filtration. Neutral lipid standards were purchased from Nu-Check-Prep, Inc., Elysian, MN. Phospholipid standards were purchased from Supelco, Inc., Bellefonte, PA. D-[1-^{14}C] glucose (specific activity, 3.0 mC/mole) was purchased from New England Nuclear, Boston, MA. All solvents used were glass distilled and purchased from Burdick and Jackson Laboratories, Muskegon, MI. Other chemicals were reagent grade or better and used without further purification.

RESULTS

Distribution of Glucose Radioactivity in HTC Cell Cultures

The distribution of glucose mass into the various fractions of the HTC cell cultures is presented in Table I. The micrograms glucose incorporated into evolved CO_2, total cell matter, and total cell lipids of serum-supplemented HTC cells were several times higher than observed for the serum-free counterparts. However, the percentages of glucose radioactivity incorporated into these fractions were higher in the case of the serum-free cultures. Most of the glucose incorporated into cells incubated in either serum-supplemented or serum-free Swim's 77 medium was localized in the nonlipid fraction. The incubation media contained the bulk of the administered glucose radioactivity. The percentage of radioactivity precipitable from the incubation media by TCA was low. Glucose radioactivity localized in media lipids was negligible, indicating that lipids were not being synthesized from D-[1-^{14}C] glucose and excreted back into the medium. DEAE-cellulose chromatography of the TCA supernatants of both 2-hr media revealed that two-thirds of the radioactivity migrated with the solvent front along with labeled glucose. However, the TCA supernatants of the 12-hr serum-supplemented and serum-free incubation media contained a much lower percentage of ^{14}C radioactivity (5% and 29%, respectively) as

TABLE I

Distribution of Glucose in HTC Cell Cultures[a]

Incubation time (hr)	Evolved CO_2	Cell fraction			Media fraction			Total recovery of ^{14}C (%)
		Total	Lipids	Nonlipids	Total	TCA ppt.	TCA soluble	
		Swim's 77 + 5% Bovine and 5% Fetal Calf Serum (2790 μg glucose/culture flask)						
1	0.8 (0.1)[b]	108.8 (3.9)	11.2 (0.4)	97.6 (3.5)	27.00.3 (96.8)	--	--	100.7
2	2.8 (0.1)	164.6 (5.9)	14.0 (0.5)	150.6 (5.4)	2694.7 (96.6)	11.2 (0.4)	2683.5 (96.2)	102.6
4	8.4 (0.3)	251.1 (9.0)	25.1 (0.9)	226.0 (8.1)	2323.7 (83.3)	--	--	92.6
8	27.9 (1.0)	332.0 (11.9)	30.7 (1.1)	301.3 (10.8)	2332.0 (83.6)	--	--	96.5
12	53.0 (1.9)	362.6 (13.0)	22.3 (0.8)	340.3 (12.2)	2387.9 (85.6)	30.7 (1.1)	2357.2 (84.5)	100.5
24	100.4 (3.6)	251.1 (9.0)	19.5 (0.7)	231.6 (8.3)	2298.6 (82.4)	--	--	95.0
		Swim's 77: Serum-free (108 μg glucose/culture flask)						
1	0.3 (0.3)	14.7 (13.6)	0.8 (0.7)	13.9 (12.9)	89.5 (82.9)	--	--	96.8
2	1.2 (1.1)	20.4 (18.9)	1.0 (0.9)	19.4 (18.0)	78.5 (72.7)	1.0 (0.9)	77.5 (71.8)	92.7
4	2.4 (2.2)	20.2 (18.7)	1.0 (0.9)	19.2 (17.8)	71.2 (65.9)	--	--	86.9
8	4.6 (4.3)	17.4 (16.1)	1.5 (1.4)	15.9 (14.7)	70.3 (65.1)	--	--	85.5
12	6.6 (6.1)	13.0 (12.0)	0.9 (0.8)	12.1 (11.2)	79.5 (73.6)	2.2 (2.0)	77.3 (71.6)	91.8
24	7.5 (6.9)	8.7 (8.1)	0.6 (0.6)	8.1 (7.5)	87.8 (81.3)	--	--	96.3

[a]Micrograms glucose distributed in various fractions of the HTC cell cultures were calculated using (i) the number of dpm's of ^{14}C incorporated into each fraction, and (ii) the specific radioactivity of glucose in each culture flask. Values are expressed per culture flask (ca. 15 x 10^6 cells at zero hours incubation). Cell numbers per culture flask at zero hours incubation, as determined using a hemocytometer, agreed to ± 6%. HTC = minimal deviation hepatoma 7288C cells.

[b]Values in parentheses refer to the percentage of glucose in each culture flask incorporated into each fraction of the HTC cell cultures. Data in this table is expressed as the mean of duplicate samples, whose values were within the following limits of agreements: CO_2: ± 10%; total cell matter: ± 10%; cell lipids: ± 15%; and total medium: ± 2%.

TABLE II

Glucose Incorporation into HTC Cell Lipids[a]

Incubation time (hr)	Total cell incorporation	Total cell lipids	Micrograms glucose incorporated[b]										
			Neutral lipids	Polar lipids	Sterols	Sterol esters	FFA	TG	PC	PE	SPH	PS	PI
Swim's 77 + 5% Bovine and 5% Fetal Calf Serum (2790 μg glucose/culture flask)													
1	109	11.2	3.1	5.1	0.74	0.09	0.56	0.67	2.54	0.67	0.38	0.21	0.89
2	165	14.0	5.9	5.3	1.73	0.17	0.77	2.09	2.04	0.79	0.56	0.39	1.00
4	251	25.1	12.5	8.9	3.69	0.41	2.07	4.87	3.52	1.05	1.15	0.68	1.69
8	332	30.7	13.6	12.3	5.96	0.50	2.87	2.87	4.56	1.33	2.05	0.72	2.27
12	363	22.3	10.2	9.1	5.71	0.60	2.87	0.74	2.92	0.61	1.72	0.57	1.83
24	251	19.5	8.4	9.2	4.98	0.67	1.13	0.88	3.59	0.92	1.59	0.77	1.71
Swim's 77: Serum-free (108 μg glucose/culture flask)													
1	14.7	0.8	0.3	0.4	0.12	0.01	0.03	0.07	0.10	0.05	0.06	0.03	0.07
2	20.4	1.0	0.3	0.6	0.12	0.01	0.03	0.11	0.12	0.04	0.14	0.04	0.09
4	20.2	1.0	0.4	0.6	0.14	0.01	0.04	0.10	0.13	0.04	0.18	0.04	0.13
8	17.4	1.5	0.2	1.0	0.10	0.01	0.02	0.05	0.34	0.06	0.33	0.05	0.21
12	13.0	0.9	0.2	0.5	0.10	0.01	0.02	0.02	0.08	0.04	0.18	0.02	0.11
24	8.7	0.6	0.2	0.3	0.06	0.02	0.01	0.03	0.05	0.02	0.13	0.02	0.11

[a]FFA = free fatty acids; TG = triglycerides; PC = phosphatidylcholine; PE = phosphatidylethanolamine; SPH = sphingomyelin; PS = phosphatidylserine; PI = phosphatidylinositol; HTC = minimal deviation hepatoma 7288C cells.

[b]Micrograms glucose incorporated into HTC cell matter and cell lipids were calculated as in Table I. Values, as in Table I, are expressed per culture flask.

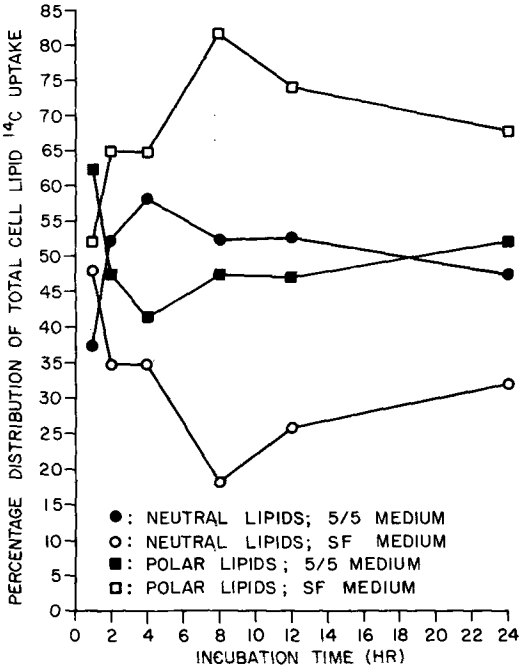

FIG. 1. Percentage of total lipid radioactivity found in neutral and polar lipid fractions of HTC cells cultured in serum-free (SF) and serum-supplemented (SS) Swim's 77 medium.

unmetabolized glucose (solvent front).

Incorporation of Glucose Radioactivity into HTC Cell Lipids

The micrograms of glucose incorporated into HTC cell total lipids, neutral and polar lipid fractions, and individual lipid classes are given in Table II. The cells cultured on serum-supplemented medium incorporated a higher amount of glucose into all lipid classes than did their serum-free counterparts as expected. In both serum-supplemented and serum-free cultures, the number of micrograms glucose incorporated into individual neutral lipid classes was as follows: sterols > triglycerides > free fatty acids > sterol esters. The amount of glucose incorporated into individual polar lipids of serum-supplemented cultures was distributed in the following manner: phosphatidylcholine > phosphatidylinositol > sphingomyelin > phosphatidylethanolamine > phosphatidylserine. Polar lipids of cells incubated in serum-free medium revealed a different pattern of ^{14}C uptake: sphingomyelin > phosphatidylcholine > phosphatidylinositol > phosphatidylethanolamine > phosphatidylserine.

Distribution of Radiolabel in Neutral Lipid and Polar Lipid Fractions

The percentage distribution of HTC cell total lipid radioactivity in neutral and polar lipid fractions at various incubation times for both serum-supplemented and serum-free cultures is depicted in Figure 1. The percentage of glucose radioactivity incorporated into polar lipids of cells cultured on serum-free medium increased (maximum at 8 hr), whereas substrate radioactivity incorporated into polar lipids of cells cultured on serum-supplemented media decreased (minimum at 4 hr). The neutral lipids exhibited reciprocal behavior.

Distribution of Radioactivity in Individual Neutral Lipid Classes

The ^{14}C incorporation profiles into individual neutral lipid classes of cells grown on

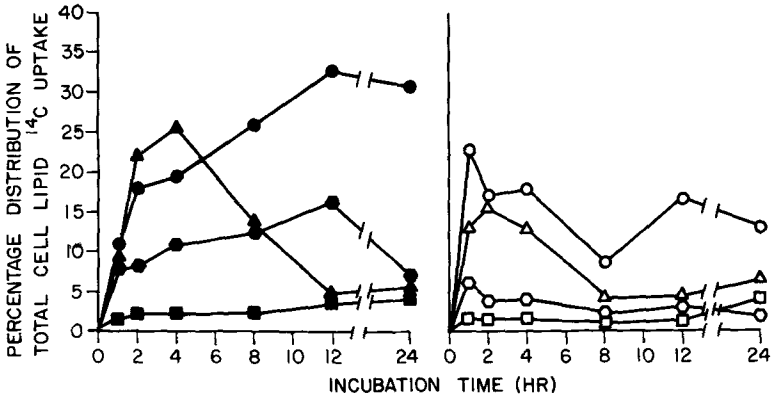

FIG. 2. Percentage of total lipid radioactivity found in individual neutral lipid classes of HTC cells cultured in serum-free and serum-supplemented Swim's 77 medium. Symbols represent: open = cultured in serum-free medium; closed = cultured in serum-supplemented medium; ● and ○ = sterols; ▲ and △ = triglycerides; ● and ○ = free fatty acids; ■ and □ = sterol esters.

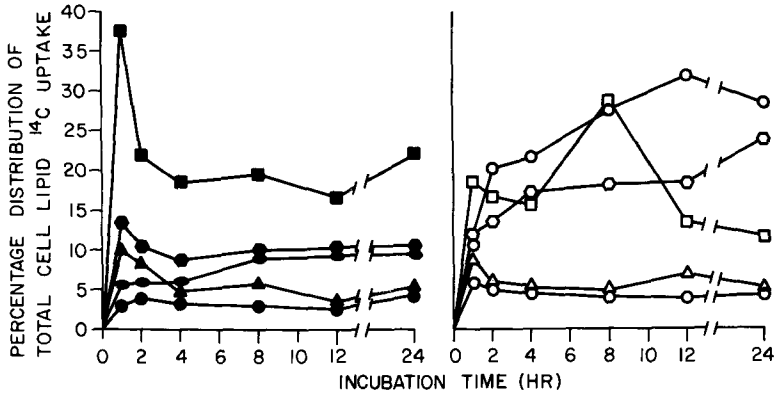

FIG. 3. Percentage of total lipid radioactivity found in individual polar lipid classes of HTC cells cultured in serum-free and serum-supplemented Swim's 77 medium. Symbols represent: open = incubation in serum-free medium; closed = incubation in serum-supplemented medium; ■ and □ = phosphatidylcholine; ● and ○ = phosphatidylinositol; ▲ and △ = phosphatidylethanolamine; ● and ○ = phosphatidylserine; ⬬ and ⬭ = sphingomyelin.

both media are shown in Figure 2. The order of radioactivity incorporation was sterols > triglycerides > free fatty acids > sterol esters in the cells grown on both media for most time periods. Triglyceride radioactivity reached a maximum between the second and fourth hour and then declined in both cases. Serum-supplemented cells contained a higher percentage of radioactivity in the free fatty acids after the first hour than did the serum-free cells. The amount of radioactivity incorporated into sterol esters remained low in cells cultured on both media despite the high percentage of radioactivity in the free sterols.

Distribution of Radioactivity in Individual Polar Lipid Classes

The incorporation of radiolabel from D-[1-^{14}C] glucose into individual HTC cell polar lipid classes is presented in Figure 3. Phosphatidylcholine contained the highest percentage of radioactivity in the serum-supplemented cultures, but not in the serum-free cultures. Except for a few time periods, phosphatidylcholine contained ca. 15-20% of the total lipid radioactivity during the 24 hr for both cultures. Phosphatidylinositol of serum-supplemented cells contained ca. 10% of the total lipid radioactivity at all time periods but the percentage of radioactivity in this lipid class steadily increased to over 20% at 24 hr in the serum-free cultures. The percentage of radioactivity incorporated into sphingomyelin differed markedly for cells cultured on the serum-free media; radioactivity of serum-supplemented cultures remained between 5-10% from 1-24 hr, but sphingomyelin radioactivity increased to over 20% at 2 hr and peaked at over 30% at 12 hr. The percentages of HTC cell total lipid radiolabel uptake due to phosphatidylethanolamine and phosphatidylserine were small throughout the 24 hr incubation period and essentially the same for both serum-supplemented and serum-free cultures.

Glucose Contribution to HTC Cell Lipid Mass

The percentage contribution of glucose to the HTC cell lipid mass of serum-supplemented cultures was calculated using: (a) lipid mass data obtained earlier (8) for HTC cell cultures grown in serum-supplemented Swim's 77 medium, (b) the number of HTC cells per culture flask, (c) the initial rate (1 hr incubation) of D-[1-^{14}C] glucose incorporation into HTC cell lipids, (d) the amount of glucose present, and (e) the doubling times for HTC cells grown in serum-supplemented medium (8). Such calculations revealed that the contribution of glucose to total HTC cell lipid mass of serum-supplemented cultures was 39.6%, while its contribution to the polar lipid and neutral lipid cell mass of these cultures was 51.1% and 37.4%, respectively. These values are probably conservative, however, due to the fact that (a) some D-[1-^{14}C] glucose radioactivity could be lost as $^{14}CO_2$ via the pentose phosphate pathway, and (b) the suboptimal level of glucose employed in the incubation medium in order to minimize dilution of the radiolabeled glucose could result in a lower velocity of glucose uptake into the HTC cells (25). The percentage contribution of glucose to the total cell lipid mass of serum-free cultures was not calculated due to their much lower glucose concentration compared to the serum-supplemented cultures.

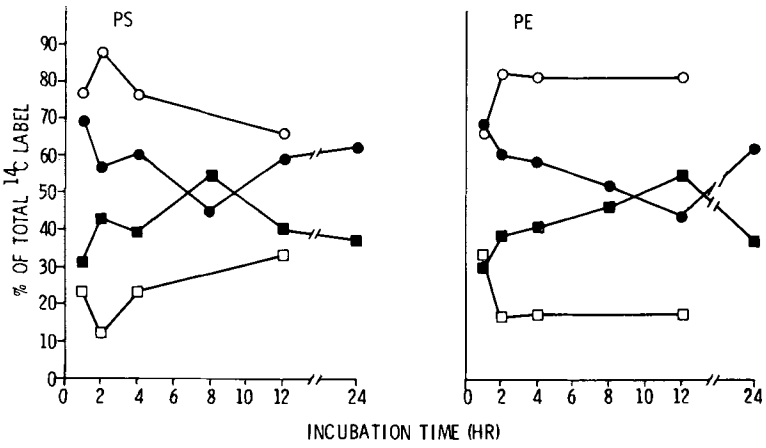

FIG. 4. Percentage distribution of radioactivity into the fatty acyl and glycerol moieties of cellular phosphatidylserine (PS) and phosphatidylethanolamine (PE) of HTC cells cultured in serum-free and serum-supplemented medium containing D-[1-^{14}C] glucose. ■ = fatty acyl moiety; serum-supplemented culture medium; ● = glycerol moiety; serum-supplemented culture medium; □ = fatty acyl moiety; serum-free culture medium; ○ = glycerol moiety; serum-free culture medium.

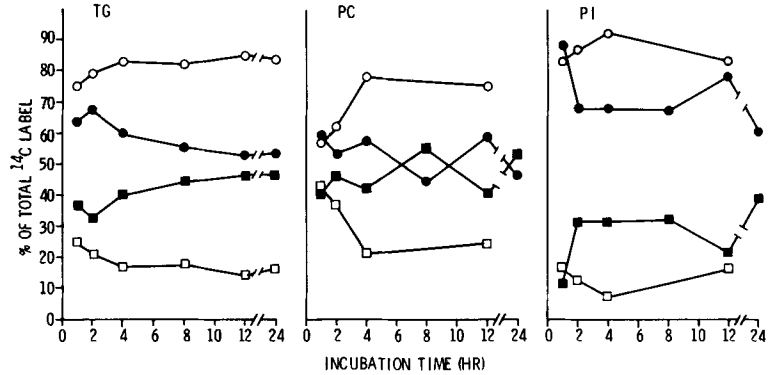

FIG. 5. Percentage distribution of radioactivity into the fatty acyl and glycerol moieties of cellular triglycerides (TG), phosphatidylcholine (PC), and phosphatidylinositol (PI) of HTC cells cultured in serum-free and serum-supplemented medium containing D-[1-^{14}C] glucose. ■ = fatty acyl moiety; serum-supplemented culture medium; ● = glycerol moiety; serum-supplemented culture medium; □ = fatty acyl moiety; serum-free culture medium; ○ = glycerol moiety; serum-free culture medium.

Radioactivity in Fatty Acyl versus Glycerol Moieties

Figures 4 and 5 depict the distribution of glucose ^{14}C in glycerol and fatty acid moieties of HTC cell triglycerides and phosphoglycerides. Each class exhibited a characteristic profile. Generally, the lipids of serum-free cultures contained a higher percentage of radioactivity in glycerol or glycerophosphoryl base moiety than did those of serum-supplemented cultures. Glycerol phosphoryl bases of cell phosphoglycerides of serum-supplemented cultures generally contained as much or more radiolabel than did the fatty acyl moieties.

DISCUSSION

The Swim's 77 medium used for this study was prepared in the normal manner except that glucose, routinely added to the medium at 1 g/liter, was omitted, and D-[1-^{14}C] glucose was substituted as the sole carbohydrate source. This was done to minimize dilution of the ^{14}C radiolabel by unlabeled glucose and maximize ^{14}C uptake. The level of glucose present in serum-supplemented cultures was due to the mass of D-[1-^{14}C] glucose added as well as the amount of glucose present in the bovine and fetal calf serum supplements to the Swim's 77

medium. The amount of glucose in such cultures, as measured enzymatically, was 2790 μg glucose/culture flask. The mass of glucose in the serum-free cultures was due entirely to the D-[1-^{14}C] glucose added and was 108 μg/culture flask. The distribution of ^{14}C radiolabel from D-[1-^{14}C] glucose into cell lipids of both serum-supplemented and serum-free HTC cell cultures was determined in this study. This was done in order to determine the effect on lipid synthesis from D-[1-^{14}C] glucose by dilution of the label via unlabeled glucose present in the serum, as well as other molecules, which conceivably could act as substrates for lipid synthesis (18,26).

The bulk of the radioactivity from D-[1-^{14}C] glucose that remained in the incubation media was neither TCA-precipitable nor lipid-extractable via the Bligh and Dyer procedure. This rules against the possibility of extensive amounts of glucose being taken up by the HTC cells and excreted back into the medium as proteins, lipids, or lipoproteins. DEAE-cellulose chromatography of the TCA supernatants of the 12-hr incubation media revealed that most (71% and 95% for serum-free and serum-supplemented media, respectively) of the ^{14}C-glucose was converted to negatively-charged molecules. In view of the above data and the high glycolytic potential of this hepatoma (27-29), the media radioactivity due to negatively-charged compounds was probably lactic acid, with the remaining media radioactivity attributable to unutilized ^{14}C-glucose. Serum-free HTC cell cultures oxidized a higher percentage of radiolabeled glucose to $^{14}CO_2$ than did the serum-supplemented cultures. However, the low level of $^{14}CO_2$ production observed with either serum-free or serum-supplemented cultures, when compared to the amount of labeled glucose converted to lactic acid, indicated that exogenous D-[1-^{14}C] glucose was not substantially channeled into the citric acid cycle or the pentose-phosphate pathway.

The percentage of D-[1-^{14}C] glucose converted to total cell lipids in serum-free cultures was 1.0-1.8 times that observed in the serum-supplemented cultures, depending on time of incubation in the different media. Substantial differences in the patterns of percentage distribution of glucose into neutral and polar lipid fractions (Fig. 1), as well as into individual lipid classes (Figs. 2,3). were observed between serum-supplemented and serum-free cultures. The selective differences in the patterns of percent distribution of glucose into individual cell lipid classes were such as to negate the possibility of a nonspecific difference in incorporation of D-[1-^{14}C] glucose into HTC cell lipids, as would be expected if differences in concentration of labeled glucose between the two incubation media were the only factors influencing glucose utilization by HTC cells.

Analysis of the fatty acyl and glycerol moieties of triglycerides and phosphoglycerides from HTC cells revealed more than 50% of the lipid radiolabel from D-[1-^{14}C] glucose was found in the glycerol moieties. This is in agreement with studies performed on Ehrlich ascites (30) and rabbit liver (31) cells using D-[U-^{14}C] glucose, but in contrast to the findings of Watson (23,32) using HTC cells with $^{3}H_2O$ and D-[U-^{14}C] glucose as the radioactive substrates. The dissimilarities between this data and that obtained by Watson may be due to differences in the culture systems and radiolabeled substrates employed. Serum-free HTC cell cultures had a much greater percentage of radiolabel in the glycerol moiety of cell glycerides than was observed for their serum-supplemented counterparts.

These findings suggest the presence of other substrates, in addition to glucose, for HTC cell lipid synthesis. In addition, the data concerning the percent contribution of glucose to HTC cell lipid mass indicates that, although glucose appears to be a substantial contributor to total HTC cell lipid mass, other precursors present in the incubation medium also furnish a substrate pool for synthesis of HTC cell lipids. Exogenous serum lipids would appear to be the most likely candidates for major contributors to HTC cell lipid mass. However, studies by Wood et al. (8-10) have demonstrated that HTC cells use serum lipids only sparingly in the synthesis of cellular lipids. Therefore, a question arises as to which moieties present in the culture system (besides glucose) serve as substrates for the synthesis of HTC cell lipids. This question is currently the subject of continuing investigations being conducted in this laboratory.

ACKNOWLEDGMENTS

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Action of Three Bile Acids on Hepatic and Intestinal Cholesterogenesis in the Rat

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ABSTRACT

Incorporation of [1 ^{14}-C] acetate into cholesterol by subcellular particles from the liver and the small intestine of rats with a biliary diversion and a duodenal perfusion of sodium taurocholate, taurochenodeoxycholate or taurodehydrocholate, was studied in vitro. In the liver, taurochenodeoxycholate prevented the increase of cholesterol synthesis induced by biliary drainage. Taurocholate had no action on cholesterol synthesis at any time, day or night. Intestinal synthesis of cholesterol was reduced by taurocholate and taurochenodeoxycholate but was not modified by taurodehydrocholate infusion.

INTRODUCTION

Cholesterol synthesis in the liver and in the small intestine of the rat was increased by biliary drainage (1,2). Conversely, the basal hepatic synthesis was decreased and the intestinal synthesis is slightly modified by a cholic acid or chenodeoxycholic acid diet (3,4). These results could be due to a direct effect of bile salts on the enzymes of cholesterol synthesis, but an indirect action is possible because of the influence of bile salts on intestinal absorption. Others have attempted to show the direct effect of bile salts using animals in which cholesterol absorption has been reduced to zero. Taurocholate only was studied in these conditions. Direct action of taurocholate on the intestinal synthesis is possible since an intestinal perfusion of taurocholate in rat with bile fistula prevents the increase of cholesterol synthesis in the jejunum and in the ileum (2). Contradictory results have been obtained regarding its influence on hepatic synthesis (5,6) in that a lymphatic diversion increases hepatic cholesterol synthesis mainly during the night (5), and cholic acid ingestion prevents this increase (5).

However, after interruption of enterohepatic circulation of bile, administration of taurocholate did not depress cholesterol synthesis (6).

A comparison of the inhibitory action of various bile salts on cholesterol synthesis during acute interruption of bile enterohepatic circulation was made. The effects of two primary bile salts, sodium taurocholate and sodium taurochenodeoxycholate, and that of sodium taurodehydrocholate (a synthetic bile salt unable to form micelles) in the rat are reported here.

METHODS

The animals used were male Wistar rats weighing from 350 to 400 g. After intallation of a biliary fistula and a duodenal catheter under ether anaesthesia, the rats were placed in restraining cages. The duodenum was perfused with isotonic saline or bile salts (1 ml/hr) and bile was collected continuously to measure bile acid secretion (7). The rats were killed by cervical dislocation 36 hr after beginning the duodenal infusion. The rats were divided into groups of five rats each, according to the time of killing and the type of duodenal perfusion.

Rats in Group A were killed at noon: Group I, controls were perfused with 0.9% NaCl. Group IIa received an infusion of Na taurocholate at 7.5 μmoles/100 g/hr. Group IIb received an infusion of Na taurocholate at 21 μmoles/100 g/hr. Group III was given an infusion of Na taurochenodeoxycholate, at 7.5 μmoles/100 g/hr.

Rats in Group B were killed at midnight: Group IV were controls and received 0.9% NaCl. Group Va were given Na taurocholate infusion, at 7.5 μmoles/100 g/hr. Group Vb were given Na taurocholate infusion, at 21 μmoles/100 g/hr. Group VI received Na taurochenodeoxycholate infusion, at 7.5 μmoles/100 g/hr. Group VII received Na taurodehydrocholate infusion, at 21 μmoles/100 g/hr.

Hepatic and intestinal cholesterol synthesis were studied by measuring the incorporation of [^{14}C] acetate into cholesterol and fatty acids in vitro. For each rat, three portions from the liver (200-300 mg each) were cut into slices with a tissue-slicer. The small intestine was removed and washed with cold saline, and subsequent operations were carried out at 0-5 C. The intestine was cut along its entire length and three fragments from the jejunum and two

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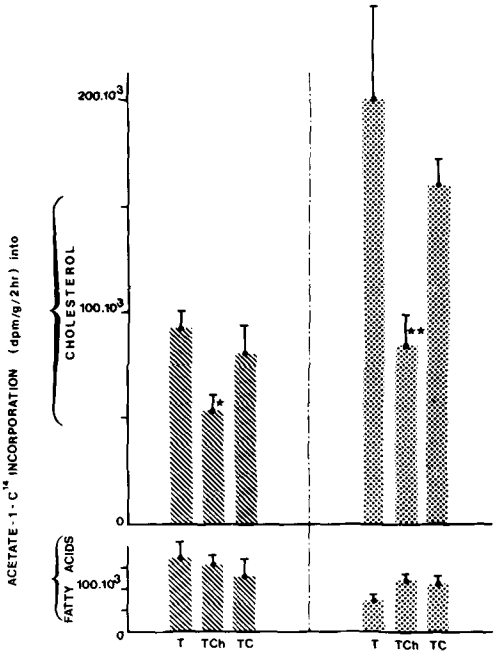

FIG. 1. Hepatic cholesterogenesis in animals with biliary diversion and intraduodenal infusion of 0.9% NaCl solution (T) sodium taurocholate (TC_1 = 7.5 μmoles/100 g/hr - TC_2 = 21 μmoles/100 g/hr), sodium taurochenodeoxycholate (TCh), sodium taurodehydrocholate (TDH: 21 μmoles/100 g/hr) for 36 hr. Rats killed at midnight:⁙; rats killed at noon///; mean values ± 1 SE are shown; student's t-test for significance (*2p<0.05 - **2p<0.01).

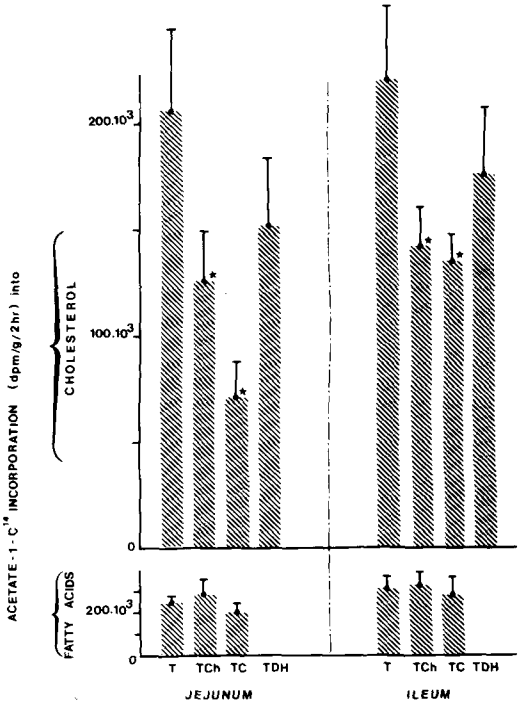

FIG. 2. Intestinal cholesterogenesis in jejunum (three fragments) and ileum (two fragments) in animals with biliary diversion and infusion of 0.9% NaCl solution, sodium taurocholate (TC), sodium taurochenodeoxycholate (TCh) and sodium taurodehydrocholate (TDH). All groups of animals were infused intraduodenally at a rate of 7.5 μmoles/100 g/hr for 36 hr. Mean values ± 1 SE are known. Student's t-test for significance (*2p<0.05 - **2p<0.01).

from the ileum were incubated for 2 hr in Krebs Ringer solution containing 1 μCi, [1-^{14}C] acetate, and 10 μmoles of unlabeled acetate in stoppered vials filled with 95% O_2, 5% CO_2. Immediately after the incubation, saponification by alcoholic potassium hydroxide was performed for 12 hr. Cholesterol was isolated by petroleum ether extraction and digitonin precipitation. The precipitate was dissolved in methanol before counting radioactivity (Intertechnique SL 40 Spectrometer). In rats infused with Na taurochenodeoxycholate (7.5 μmoles/100 g/hr), the mevalonate incorporation (0.75 μCi) into digitonin precipitable sterols was measured by the same method, under identical conditions.

RESULTS AND DISCUSSION

Hepatic cholesterogenesis: in rats infused with sodium taurocholate, acetate incorporation into cholesterol was the same as in controls both day and night (Fig. 1). Taurodehydrocholate was also without significant effect. Conversely, there was a significant difference between controls and taurochenodeoxycholate-infused rats at both times studied. However, control experiments showed that the inhibitory effect of taurochenodeoxycholate was not sufficient to reduce cholesterol synthesis to the levels observed in intact animals.

Intestinal cholesterogenesis: Figure 2 shows that taurocholate and taurochenodeoxycholate prevented the increase of cholesterol synthesis during biliary drainage. In the jejunum, taurocholate was more inhibitory than taurochenodeoxycholate. Both bile acids had the same action on ileal synthesis, but taurodehydrocholate and dehydrocholate had no action.

This work shows that the effects on cholesterol synthesis by bile acids are related to their structures. These effects are different on liver and intestinal cholesterogenesis. The observed differences probably reflect true differences between liver and intestinal cholesterogenesis. While this work was in progress, it has been

shown that exogenous [^{14}C] acetate may be significantly diluted by endogenous acetate and that some steps prior to HMG CoA reductase may be partially rate-limiting for exogenous acetate incorporation into cholesterol (8). However, these limitations prevent the assessment of absolute rates of cholesterogenesis with acetate more than the study or relative changes of cholesterogenesis which we studied. Other results obtained by measuring acetate incorporation have been entirely confirmed in more recent work using measurements of hydroxymethyl-glutarylcoenzyme A reductase (9). In the present study, the rate of acetate incorporation into fatty acids was similar in the several groups of rats. This suggests that bile acid treatment modifies neither the specific radioactivity of the acetate precursor pool nor the formation of acetylcoenzyme A (Figs. 1 and 2). Therefore, taurocholate seems to exert a direct inhibitory effect on intestinal but not on hepatic cholesterogenesis. The lack of effects of sodium taurocholate on cholesterol synthesis in the liver is not dependent on diurnal variations of this synthesis in the rat. Our results are consistent with the work of Weis and Dietschy (6) showing a lack of direct action of taurocholate on hepatic cholesterogenesis, which differs from the studies of Hamprecht et al. (5). In our study, the rate of bile salts infusion represents the normal flow of bile acids in the rat. In the study of Hamprecht et al., cholic acid was administered to rats with an intact enterohepatic circulation of bile acids, inducing supranormal delivery of bile acids to the liver. The highest dose used in our study for taurocholate infusion (21 μmoles/100 g/hr) represents the flux of bile acids during normal enterohepatic circulation in rats as calculated by Shefer et al. (10). This rate of bile acid is able to reduce to basal levels bile acid synthesis during acute biliary drainage. It seems, therefore, that taurocholate has no direct influence on cholesterol hepatic synthesis.

There is a difference between these results and those obtained during taurocholate chronic administration which depresses the HMG CoA reductase activity of the liver (3). This difference could be due to the supranormal bile acids pool which demonstrates a nonphysiological action of taurocholate. Other mechanisms may explain why cholesterol synthesis is depressed by diets containing cholic acid. This diet may increase cholesterol absorption because further addition of sitosterol prevents the effects of taurocholate (4), or prolonged inhibition of cholesterol oxidation may increase the cholesterol content of hepatic cells.

Unlike taurocholate, taurochenodeoxycholate decreases cholesterogenesis. This effect is not dependent on a change of caloric intake during taurochenodeoxycholate administration because rats which received no food during intestinal perfusion with either bile acid and control rats receiving 13 cal/24 hr (two experiments and two controls) gave essentially the same results. The effect of taurochenodeoxycholate on acetate incorporation into cholesterol could be due to a toxic effect for taurochenodeoxycholate has an inhibitor effect on the HMG CoA reductase in vitro (5). In the isolated perfused rat liver, it induces a decrease of bile flow related to a fall of hepatic ATP concentration (11). Moreover, a rate of 21 μmoles/100 g/hr induces a decrease of bile flow resulting in the death of most of the rats. There is no evidence of toxicity with a low rate of taurochenodeoxycholate infusion (7.5 μmoles/100 g/hr). Total bile flow and the fraction of bile flow independent of bile salts are not decreased. The incorporation of mevalonate into cholesterol is not modified, suggesting a specific effect of taurochenodeoxycholate on the HMG CoA reductase.

Some authors have observed that there is no direct effect of taurochenodeoxycholate on cholesterol synthesis in isolated perfused rat liver (12). This suggests that in our conditions the effects of taurochenodeoxycholate are due to an action on enzyme synthesis rather than an inhibition of the enzymes. A similar effect is also observed during chronic administration of taurochenodeoxycholate with or without sitosterol addition (3). Even if the inhibition of acetate incorporation into cholesterol is not due to a toxic effect of bile acids, this does not prove that taurochenodeoxycholate plays a major role in regulation of cholesterol synthesis in normal animals. In our work, cholesterol synthesis was not reduced to base levels during taurochenodeoxycholate infusion even if the perfusion rate (7.5 μmoles/100 g/hr) exceeds the normal secretion rate of the bile acid in bile.

For intestinal synthesis, the well known action of taurocholate is confirmed by our work. Sodium taurochenodeoxycholate had a similar but lesser effect on jejunal cholesterol synthesis. Sodium taurodehydrocholate was without action. Because dehydrocholate does not form micelles at concentrations used in our experiments, the inhibitory effect of bile salts on intestinal cholesterogenesis may depend on their detergent properties.

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Tissue Lipid Responses to 3-Hydroxy-3-Methylglutaric Acid[1] with Different Dietary Fats[2]

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ABSTRACT

Simultaneous administration of 3-hydroxy-3-methylglutaric acid (HMG) for 4 weeks to rats fed 20% saturated fats prevented rise of serum cholesterol, triglycerides, and phospholipids. Except phospholipids, other liver lipids were significantly decreased. HMG administration for 4 weeks along with atherogenic diet significantly decreased cholesterol and phospholipids of serum, liver, aorta, and heart. The phospholipids of epididymal fat and brain were also significantly lowered. The triglyceride levels in serum, liver, and epididymal fat were significantly decreased. The maximal hypolipidemic effect of HMG was observed in serum.

[1]U.S. Patent No. 3629449, dated December 21, 1971, on "Process of combatting hypercholesterolemia."

[2]A part of the work submitted to Aligarh Muslim University for Ph.D. degree.

INTRODUCTION

3-Hydroxy-3-methylglutaric acid (HMG) has been reported to reduce cholesterol, triglyceride, and phospholipid levels in rats (1-6), rabbits (7,8), and men (6,9). In these studies, hyperlipidemic conditions were either induced with 5% saturated fat, 2% cholesterol, and 1% cholic acid (2) or by other well known methods, i.e., 2% cholesterol in 6% heated corn oil (7), cholesterol and vitamin D_2 in olive oil (4,10), high carbohydrate diets (3), alcohol (6), and Triton WR-1339 (5). Since consumption of saturated fats is known to contribute to the development of coronary heart diseases (11-13), it was of more practical value to know the effect of HMG on tissue lipids of animals kept on high levels of different dietary fats. To gain knowledge regarding the tissue(s) exhibiting maximal hypolipidemic effect of HMG, the action of compound on lipid levels of various tissues of atherogenic diet-fed rats was also investigated.

MATERIALS AND METHODS

Adult male albino rats (stock colony of Indian Veterinary Research Institute, Izzatnagar, India) weighing about 120-180 g were maintained on basal diet (supplied by Hindustan Lever Ltd., India) and water ad libitum. The animals in the treated groups received one standard dose of 20 mg HMG (Schwarz/Mann, Orangeburg, NY) per kg body weight per day intraperitoneally in 1 ml normal saline. At the end of each experiment, the overnight fasted

TABLE I

Composition of High Fat Diet (18)

Ingredients	Percent by weight
Casein	21.10
Sucrose	38.45
Cellulose	16.45
Salt mixture (26)	4.00
Test fat	20.00
Vitamin mixture (27)	++

TABLE II

Total Body Weight Gain and Wet Weight of Liver of Animals Kept on High Fat and Atherogenic Diet with and without HMG[a] Treatment

Diets	Control group		HMG-treated group	
	Weight of liver	Total weight gain/rat	Weight of liver	Total weight gain/rat
1. High fat diets				
Lard	5.8 ± 0.2[b]	60 ± 4	5.2 ± 0.3	48 ± 5
Butter	6.2 ± 0.4	85 ± 5	5.8 ± 0.5	68 ± 6
Hydrogenated vegetable oil	5.7 ± 0.3	53 ± 4	5.3 ± 0.3	40 ± 2
2. Atherogenic diet	8.2 ± 0.3	50 ± 2	7.6 ± 0.7	40 ± 2

[a]HMG = 3-hydroxy-3-methylglutaric acid.

[b]Mean ± Standard Error expressed in grams.

animals were anesthetized with ether, blood was withdrawn by cardiac puncture, and serum obtained by centrifugation. The various tissues were immediately removed, washed, blotted, weighed, and frozen for subsequent analysis. The tissue lipids were extracted by the method of Folch et al. (14). Cholesterol (15), triglyceride (16), and phospholipid (17) levels were analyzed in serum and tissue extracts. Statistical significance was calculated by student's t-test.

To study the effect of HMG in preventing rise of serum and liver lipid parameters, animals in groups of five caged separately were kept on diets high in saturated fats, e.g., lard, butter, and hydrogenated vegetable oil as shown in Table I (18). The animals in treated groups received standard dose of HMG for 4 wk, while the control groups were fed the same diets and injected an equal volume of saline for the same period.

To investigate the protective effect of HMG on hyperlipidemic rats, hyperlipidemia was induced by feeding an atherogenic diet (2% cholesterol dispersed in 6% heated corn oil mixed with basal diet) (19). The animals in the treated group simultaneously received standard doses of HMG, while the control group was fed the same diet and injected an equal volume of saline. At the end of 4 wk, blood was withdrawn, and liver, aorta, heart, epididymal fat, brain, small intestine, kidney, spleen, testes, and lung were removed.

RESULTS

The animals in all experiments tolerated the treatment relatively well. The average weight gain in HMG-treated groups was significantly lower ($P<0.05$) than in their respective control groups (Table II). The magnitude of decrease in weight gain was more or less similar under all dietary conditions. However, the decrease in liver weight was insignificant. The dietary intake of HMG-treated and control group was the same.

High Fat Diets

Table III shows the effect of HMG on cholesterol, triglyceride, and phospholipid levels of serum and liver of rats fed 20% saturated fats for 4 wk. Regardless of the type of dietary fats, all lipid parameters, except liver phospholipids, were significantly decreased in serum and liver. In butter-fed rats, the effect of HMG on serum and liver lipids was more marked when compared to lard and hydrogenated vegetable oil-fed rats. The magnitude of decrease in lipid parameter in lard and hydrogenated vegetable

TABLE III

Effect of HMG[a] on Serum and Liver Lipids of Rats Receiving High Fat Diets

Dietary status	Serum lipids (mg/100 ml)			Liver lipids (mg/g)		
	Cholesterol	Triglycerides	Phospholipids	Cholesterol	Triglycerides	Phospholipids
Lard						
Control group	76 ± 3[b]	72 ± 2	166 ± 3	3.4 ± 0.1	9.2 ± 0.6	24.6 ± 1.8
HMG-treated group	60 ± 4	53 ± 2	140 ± 4	2.5 ± 0.2	6.9 ± 0.5	23.0 ± 1.7
Percent reduction	21 ($P<0.01$)	26 ($P<0.001$)	15 ($P<0.05$)	26 ($P<0.001$)	25 ($P<0.01$)	7 (N.S.)[c]
Butter						
Control group	85 ± 3	107 ± 3	199 ± 9	4.0 ± 0.2	11.4 ± 1.0	29.6 ± 2.5
HMG-treated group	56 ± 3	62 ± 2	163 ± 5	2.7 ± 0.2	6.8 ± 0.5	26.1 ± 1.8
Percent reduction	34 ($P<0.001$)	42 ($P<0.001$)	18 ($P<0.02$)	33 ($P<0.01$)	40 ($P<0.001$)	12 (N.S.)
Hydrogenated vegetable oil						
Control group	71 ± 4	81 ± 4	206 ± 9	2.9 ± 0.1	8.8 ± 0.7	26.7 ± 1.9
HMG-treated group	50 ± 3	61 ± 3	173 ± 7	2.1 ± 0.2	6.1 ± 0.3	24.4 ± 1.6
Percent reduction	29 ($P<0.001$)	27 ($P<0.01$)	16 ($P<0.05$)	27 ($P<0.001$)	31 ($P<0.01$)	9 (N.S.)

[a]HMG = 3-hydroxy-3-methylglutaric acid.
[b]Mean ± Standard Error expressed for five rats.
[c]N.S. = not significant.

TABLE IV

Effect of HMG[a] on Tissue Lipids of Rats Receiving Atherogenic Diet

Lipids	Serum, mg/100 ml	Liver, mg/g	Aorta, mg/g	Heart, mg/g	Epididymal fat, mg/g	Small intestine, mg/g	Brain, mg/g	Kidney, mg/g	Spleen, mg/g	Testes, mg/g	Lung, mg/g
Cholesterol											
Control group	401 ± 12[b]	11.5 ± 0.1	2.4 ± 0.2	1.8 ± 0.2	1.4 ± 0.1	5.7 ± 0.9	14.5 ± 1.0	3.8 ± 0.3	6.2 ± 0.5	1.4 ± 0.2	3.2 ± 0.2
HMG-treated group	261 ± 20[c] (35)[d]	8.0 ± 0.2[e] (31)	1.8 ± 0.1[e] (29)	1.2 ± 0.1[e] (33)	1.3 ± 0.1[f] (6)	4.9 ± 0.5[f] (14)	12.2 ± 1.3[f] (16)	3.7 ± 0.2	6.5 ± 0.5	1.2 ± 0.3[f] (14)	3.4 ± 0.1
Triglycerides											
Control group	290 ± 50	30.8 ± 1.8	5.4 ± 0.5	11.6 ± 1.3	107.0 ± 5.7	8.2 ± 0.8	n.d.[h]	4.5 ± 0.8	3.2 ± 0.3	5.6 ± 0.6	3.4 ± 0.8
HMG-treated group	239 ± 18[g] (40)	20.1 ± 1.9[g] (35)	4.4 ± 0.9[f] (18)	10.2 ± 1.2[f] (12)	79.0 ± 2.6[c] (26)	7.4 ± 1.1[f] (10)	n.d.	4.2 ± 0.8[f] (7)	3.0 ± 0.5[f] (6)	5.8 ± 0.7	3.3 ± 0.4
Phospholipids											
Control group	436 ± 20	43.1 ± 6.1	11.8 ± 0.8	23.4 ± 1.2	18.3 ± 2.0	11.4 ± 1.9	43.3 ± 2.9	23.2 ± 1.4	33.5 ± 2.6	13.1 ± 2.5	20.8 ± 1.4
HMG-treated group	330 ± 18[c] (24)	30.5 ± 1.0[c] (29)	9.4 ± 1.0[g] (20)	22.1 ± 2.8[f] (6)	11.0 ± 1.8[c] (40)	9.3 ± 2.6[f] (18)	35.0 ± 1.5[g] (19)	21.1 ± 2.8[f]	30.9 ± 4.5[f] (8)	13.0 ± 2.0	21.0 ± 1.3

[a]HMG = 3-hydroxy-3-methylglutaric acid.
[b]Mean ± Standard Error expressed for six rats.
[c]Significantly different from control group, $P < 0.001$.
[d]Values in parentheses indicate percent reduction with respect to control group.
[e]$P < 0.05$.
[f]Not significant.
[g]$P < 0.02$.
[h]n.d. = not determined.

oil-fed rats was the same.

Induced-Hyperlipidemic Group

The data shown in Table IV summarize the effect of 4 wk HMG treatment on hyperlipidemic rats. On HMG treatment, cholesterol was significantly decreased to the extent of 35, 31, 29, and 33%, respectively, in serum, liver, aorta, and heart. It had either none or insignificant effect on cholesterol levels of epididymal fat, intestine, brain, kidney, spleen, testes, and lung. The triglyceride levels in serum, liver, and epididymal fat were significantly lowered to the extent of 40, 35, and 26%, respectively. However, little or no decrease was observed in aorta, heart, intestine, kidney, spleen, testes, and lung. In treated groups, the phospholipid levels of serum, liver, aorta, epididymal fat and brain were significantly lowered to the extent of 24, 29, 20, 40, and 19%, respectively. The phospholipid levels of heart, intestine, kidney, spleen, testes, and lung were not significantly effected by HMG treatment.

DISCUSSION

Several studies have indicated that consumption of saturated fats contributes to the development of coronary heart diseases in the human population (11-13). Saturated fats also increase severity of atherosclerosis in animals (20-23). The present investigation shows that HMG decreased the hyperlipidemic effect of heated corn oil and cholesterol in rats. It also prevented the rise of serum and liver lipid levels of rats fed high percentage of lard, butter, and hydrogenated vegetable oil. The marked lipid lowering effect of HMG in serum and liver of butter-fed rats is interesting in view of the fact that affluent persons more prone to coronary heart disease consume larger amounts of butter. In agreement with the earlier findings in rabbits (7), HMG had a significant lipid lowering effect on serum and aorta of rats fed atherogenic diet (Table IV). In contrast, the rat liver phospholipids were significantly decreased. Among the investigated tissues, maximal hypolipidemic effect of HMG was observed in serum. As decrease in lipid parameters in one tissue was not accompanied by increase in other tissues, the effect of HMG on degradation of lipids is more likely. Since HMG is administered along with diets, it is quite likely that obesity induced by either high fat or atherogenic diet is counteracted by HMG resulting in decreased weight gain in HMG treated animals.

As evident from the results and also in accord with earlier reports (1-9), the reduction on HMG treatment was more marked in tri-

glyceride levels in rats fed either high fat or atherogenic diets. This suggests that HMG in some way causes a shift in lipoprotein spectrum which may also partially explain decreased cholesterol deposition in liver. Like nicotinic acid, cholestyramine and pyrazonic acid, HMG was unable to overcome orotic acid-induced fatty liver changes in rats (24). Therefore, the possibility of HMG inhibiting the synthesis of lipoproteins (very low density and low density) rather than releasing lipoproteins is more likely. This is also evident from the observations that the decrease in serum lipids was not accompanied by a rise of liver lipids.

Addition of fats, with and without cholesterol or cholesterol alone, to the diet is known to induce hyperlipidemia which may lead to produce atherosclerotic conditions (8,10-13,20-23). Unlike p-chlorophenoxyisobutyrate (CPIB) (18), HMG decreased serum cholesterol levels of rats fed high percentage of saturated fats. The present investigation, therefore, strengthens the belief that HMG has an antihyperlipidemic effect regardless of the type of dietary fats ingested. As LD_{50} of HMG is above 1000 mg/kg in mice (25), further toxicological studies are desirable in primates before clinical studies are undertaken to establish its therapeutic value.

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Serum and Liver Lipid Responses to 3-Hydroxy-3-methylglutaric Acid[1] in Rats on Different Carbohydrate Diets[2]

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ABSTRACT

Groups of male adult albino rats were administered 3-hydroxy-3-methylglutaric acid (HMG) intraperitoneally along with six diets differing only in the type of carbohydrate used. Groups not treated with HMG served as controls. HMG showed a significant cholesterol- and triglyceride-lowering effect in the whole serum, serum β-lipoproteins, and liver of animals on all types of dietary carbohydrates. The effect was more marked in glucose, fructose, sucrose, and lactose. The phospholipid levels in whole serum, serum β-lipoproteins, as well as in liver were also significantly lowered on all types of carbohydrates except dextrin and starch fed animals, where it had no effect on liver levels. The lipid-lowering effect of HMG seems to be independent of the type of carbohydrate in diet.

INTRODUCTION

There has been a growing suspicion that dietary carbohydrates by virtue of an influence on the serum triglycerides and phospholipids may play a role in the development of atherosclerosis (1-3). The types of carbohydrate in the diet are known to have a material influence on serum lipids (4-8).

3-Hydroxy-3-methylglutaric acid (HMG) is effective against hyperlipidemia, more particularly hypertriglyceridemia in rats (9-14), rabbits (15,16), and men (13,17). Since the endogenous synthesis of triglycerides is influenced by the type of carbohydrates in food (18-20), it was of interest to study the relative effect of HMG in lowering serum and liver lipids when given with different carbohydrate regimes. The present work describes the effect of HMG on cholesterol, triglyceride, and phospholipid levels in whole serum, serum β-lipoproteins, and liver of rats fed diets containing either glucose, fructose, sucrose, lactose, starch, or dextrin as the only source of carbohydrates.

MATERIALS AND METHODS

Sixty male adult albino rats (stock colony of Indian Veterinary Research Institute, Izzatnagar, India) weighing about 130-170 g were randomly and equally divided into six groups. They were kept on a synthetic diet containing either glucose, fructose, sucrose, lactose, starch (corn), or dextrin. The laboratory mixed food (21) given ad libitum for a period of 4 wk consisted of 72% carbohydrates, 20% casein, 2% cellulose, 1% corn oil, 0.5% dl-methionine, 0.1% mannitol, 4% salt mixture (22), and a vitamin mixture.

Along with the diet, five animals in each group received each day intraperitoneally HMG (Schwarz/Mann, Orangeburg, NY) at a concentration of 20 mg per kg body weight in 1 ml saline. The remaining five animals in each group receiving equal volumes of saline served as

[1]U.S. Patent No. 3629449, dated December 21, 1971, on "Process of combatting hypercholesterolemia."

[2]A part of the work submitted to Aligarh Muslim University for Ph.D. degree.

TABLE I

Average Body Weight Gain of Carbohydrate-Fed Rats with and without (Control) HMG[a]-Treatment

Dietary status	Average weight gain for 4-wk period (g)		
	Control group	HMG-treated group	Significance
Basal	40 ± 5	-	-
Glucose	50 ± 4	40 ± 4	$P < 0.05$
Fructose	52 ± 6	35 ± 4	$P < 0.01$
Sucrose	58 ± 3	34 ± 3	$P < 0.01$
Lactose	45 ± 5	36 ± 4	$P < 0.02$
Starch (corn)	30 ± 3	21 ± 2	$P < 0.01$
Dextrin	25 ± 2	19 ± 1	$P < 0.05$

[a]HMG = 3-hydroxy-3-methylglutaric acid.

paired control. One group of five animals was fed a normal basal diet (supplied by Hindustan Lever Ltd., India) for reference purpose. The weight and diet consumption of animals were recorded every week. The overnight fasted animals were anesthetized with ether, blood was withdrawn by cardiac puncture, and serum obtained. Serum β-lipoproteins were separated by the dextran sulfate precipitation method (23). Liver lipids were extracted by the method of Folch et al. (24). Cholesterol (25), triglyceride (26), and phospholipid (27) levels were analyzed in whole serum, serum β-lipoproteins, and liver. Statistical significance was calculated by student's t-test.

RESULTS

At the termination of study, we observed that the average weight gain by HMG-treated animals was lower than control animals. The difference in weight gain due to HMG treatment was statistically significant with all diets as shown in Table I. There was no marked difference in dietary intake of control and HMG-treated animals. In both groups, the animals were active throughout the period of investigation. The study confirms the earlier findings (20,28,29) that as compared to polysaccharides, diets containing mono- and disaccharide increased cholesterol, phospholipid, and triglyceride levels more in serum and liver (Tables II-IV). Administration of HMG for 4 wk lowered cholesterol, triglyceride, and phospholipid levels in serum and liver on all type of carbohydrate in diets. The decrease in cholesterol and triglyceride levels in serum and liver on HMG treatment was significantly more marked in glucose, fructose, sucrose, and lactose than in dextrin and starch fed rats (Tables II-IV). On HMG treatment, the serum and liver phospholipid levels were significantly lowered to a lesser extent than were cholesterol and triglyceride levels. However, the level remained insignificant in livers of dextrin and starch fed rats (Table IV). The lipids of serum β-lipoproteins were also lowered on HMG treatment on all types of carbohydrate in diet. The extent of magnitude in lowering cholesterol, triglyceride, and phospholipid levels remained approximately similar in whole serum and serum β-lipoproteins (Tables II-IV).

DISCUSSION

Dietary carbohydrates are known to increase serum and liver lipids (4-8,28,29). Except liver phospholipid of starch- and dextrin-fed rats, HMG significantly lowered serum and liver cholesterol, triglyceride, and phospholipid

TABLE II

Effect of HMG[a] on Serum and Liver Cholesterol of Rats Fed High Carbohydrate Diets

Dietary status	Whole serum (mg/100 ml)		Serum β-lipoproteins (mg/100 ml)		Liver (mg/g)	
	Control group	HMG-treated group	Control group	HMG-treated group	Control group	HMG-treated group
Basal	62 ± 2	-	36 ± 3	-	2.4 ± 0.1	-
Glucose	115 ± 10[b]	78 ± 5[c] (32)[d]	76 ± 7	52 ± 4[c] (32)	3.8 ± 0.3	2.2 ± 0.2[e] (42)
Fructose	110 ± 5	76 ± 5[e] (31)	76 ± 8	49 ± 4[c] (35)	5.2 ± 0.2	3.2 ± 0.2[e] (38)
Sucrose	124 ± 8	78 ± 6[e] (37)	86 ± 5	61 ± 5[c] (29)	4.2 ± 0.3	2.5 ± 0.2[e] (40)
Lactose	115 ± 8	71 ± 6[e] (38)	94 ± 7	58 ± 6[c] (38)	3.7 ± 0.2	2.3 ± 0.2[e] (38)
Starch (corn)	96 ± 8	72 ± 4[f] (25)	62 ± 8	50 ± 6[f] (19)	3.0 ± 0.1	2.3 ± 0.2[f] (23)
Dextrin	95 ± 7	79 ± 5[f] (17)	70 ± 6	55 ± 5[f] (21)	2.8 ± 0.2	2.1 ± 0.2[f] (25)

[a]HMG = 3-hydroxy-3-methylglutaric acid.
[b]Mean ± Standard Error expressed for five rats.
[c]$P < 0.01$.
[d]Values in parentheses indicate percent reduction with respect to control group.
[e]Significantly different from control group $P < 0.001$.
[f]$P < 0.02$.

TABLE III

Effect of HMG[a] on Serum and Liver Triglycerides of Rats Fed High Carbohydrate Diets

Dietary status	Whole serum (mg/100 ml)		Serum β-lipoproteins (mg/100 ml)		Liver (mg/g)	
	Control group	HMG-treated group	Control group	HMG-treated group	Control group	HMG-treated group
Basal	58 ± 4	-	45 ± 3	-	6.2 ± 0.2	-
Glucose	131 ± 4[b]	86 ± 5[c] (34)[d]	102 ± 7	67 ± 6[c] (34)	11.7 ± 1.3	8.1 ± 0.6[e] (31)
Fructose	142 ± 5	80 ± 3[c] (44)	111 ± 3	66 ± 4[c] (41)	18.7 ± 2.0	11.1 ± 1.0[e] (41)
Sucrose	136 ± 3	89 ± 5[c] (35)	99 ± 4	65 ± 3[c] (34)	14.1 ± 1.7	8.3 ± 0.8[e] (41)
Lactose	123 ± 5	74 ± 5[c] (40)	96 ± 7	57 ± 4[c] (41)	12.3 ± 1.2	7.0 ± 0.8[e] (43)
Starch (corn)	90 ± 5	70 ± 6[e] (20)	69 ± 4	53 ± 4[e] (23)	9.1 ± 0.9	6.9 ± 0.6[f] (24)
Dextrin	85 ± 8	67 ± 5[g] (21)	65 ± 5	50 ± 6[g] (23)	8.9 ± 0.4	6.5 ± 0.3[f] (27)

[a]HMG = 3-hydroxy-3-methylglutaric acid.
[b]Mean ± Standard Error expressed for five rats.
[c]Significantly different from control group $P < 0.001$.
[d]Values in parentheses indicate percent reduction with respect to control group.
[e]$P < 0.01$.
[f]$P < 0.05$.
[g]$P < 0.02$.

levels on all type of carbohydrates. The difference in serum and liver cholesterol and triglyceride levels in control and HMG-treated animals was striking. Statistical analysis of data showed that HMG had more significant lowering effect on cholesterol and triglyceride levels of serum and liver of mono- and disaccharide fed rats. It is interesting to note that unlike p-chlorophenoxyisobutyrate (CPIB) (30), HMG prevents sucrose-induced hypertriglyceridemia. The failure of treated animals to gain weight normally could imply that the lipid lowering effect of HMG may be nonspecific and due to reduced food absorption. The least weight gain in starch- and dextrin-fed animals is also reflected in comparatively less rise in all lipid parameters in serum and liver. Since HMG is administered simultaneously along with the diets, it is quite likely that obesity induced by high carbohydrate diets is counteracted by HMG resulting in decreased weight gain in HMG treated animals.

High carbohydrate diets are known to enhance very low density lipoprotein (VLDL) synthesis as well as secretion of VLDL triglycerides by increasing the contribution of liver triglycerides (6,31,32). The HMG-induced lowering of lipid parameters in serum β-lipoproteins and also in liver could be due either to inhibition of VLDL synthesis or to VLDL triglyceride release in liver. It has been shown that HMG failed to prevent orotic acid-induced fatty liver (33). This may rule out the possibility of HMG inhibiting the release of VLDL and low density lipoproteins (LDL). Since lipoproteins participate in mobilization of liver lipids (34,35), and HMG significantly lowers lipid parameters of animals receiving high carbohydrates in diet, it appears that HMG checks VLDL synthesis rather than release of VLDL. The decrease in serum β-lipoproteins in human (13) and rats (11) caused by HMG treatment has also been reported. In light of these findings, it appears that hyperlipoproteinemia (7,36,37) and hyperlipidemia (28,29) induced by dietary carbohydrates could be prevented by HMG treatment. In addition to inhibiting cholesterol biosynthesis (38,39), HMG like CPIB may interfere at some stage of fatty acid synthesis (40).

In recent years, much interest has been focused upon the different kinds of carbohydrates in hyperlipoproteinemia (7,36,37). As HMG is capable of regressing the rise in serum and liver lipids of animals regardless of the type of carbohydrates ingested, particularly sucrose, the potential of HMG as hypolipidemic agent becomes significant.

ACKNOWLEDGMENTS

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TABLE IV

Effect of HMG[a] on Serum and Liver Phospholipids of Rats Fed High Carbohydrate Diets

Dietary status	Whole serum (mg/100 ml)		Serum β-lipoproteins (mg/100 ml)		Liver (mg/g)	
	Control group	HMG-treated group	Control group	HMG-treated group	Control group	HMG-treated group
Basal	108 ± 5	-	47 ± 4	-	18.2 ± 0.7	-
Glucose	219 ± 8[b]	176 ± 5[c] (20)[d]	80 ± 4	63 ± 3[e] (22)	31.1 ± 1.0	27.4 ± 1.1[f] (12)
Fructose	234 ± 14	176 ± 11[f] (25)	92 ± 3	70 ± 4[c] (24)	38.5 ± 1.8	29.2 ± 1.9[e] (24)
Sucrose	224 ± 5	156 ± 7[c] (30)	98 ± 5	67 ± 4[c] (32)	34.1 ± 0.7	28.5 ± 0.9[c] (17)
Lactose	256 ± 12	208 ± 6[f] (19)	110 ± 9	86 ± 8[f] (22)	36.2 ± 1.0	29.4 ± 1.6[e] (20)
Starch (corn)	202 ± 8	160 ± 6[e] (21)	85 ± 4	67 ± 6[e] (21)	27.5 ± 2.8	24.2 ± 2.6[g] (12)
Dextrin	204 ± 9	158 ± 7[e] (23)	80 ± 2	60 ± 7[c] (25)	24.2 ± 2.5	22.1 ± 1.9[g] (9)

[a]HMG = 3-hydroxy-3-methylglutaric acid.
[b]Mean ± Standard Error expressed for five rats.
[c]Significantly different from control group $P < 0.001$.
[d]Values in parentheses indicate percent reduction with respect to control group.
[e]$P < 0.01$.
[f]$P < 0,02$.
[g]Not significant.

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Involvement of Cytochrome b_5 in the Oxidative Desaturation of Linoleic Acid to γ-Linolenic Acid in Rat Liver Microsomes[1]

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ABSTRACT

The effects of antibodies against microsomal electron-transport components on the in vitro activity of Δ^6-desaturation of linoleic acid to γ-linolenic acid have been studied in intact microsomal membranes of rat liver. Reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate (NADPH) (0.87 mM) served as electron donors, and effectively prompted the Δ^6-desaturase activities with yields of about 1.1 to 1.3 nmol per mg of protein in 10 min. Of the two antibodies studied under the same in vitro conditions, i.e., rabbit antisera preparations against rat liver microsomal hydrophilic parts of cytochrome b_5 and NADPH-cytochrome c reductase, only the antibody against cytochrome b_5 demonstrated a marked ability to inhibit the Δ^6-desaturase activity. This evidence supports a participation of cytochrome b_5 in the Δ^6-desaturation of linoleic acid and suggests a pathway analogous to the Δ^9-desaturation of stearyl-CoA.

INTRODUCTION

Several reports have been made to provide evidence that Δ^6-desaturase (e.g., 18:2→18:3 ω6) is different from Δ^9-desaturase (e.g. 18:0→18:1 ω9) by experiments of dietary induction, circadian rhythm (1), and hepatomas with different growth rates (2). Among the various desaturations of fatty acids catalyzed by liver microsomes, Δ^9-desaturation of stearyl-CoA to oleyl-CoA has been shown to consist of NADH-cytochrome b_5 reductase, cytochrome b_5 (cyt. b_5) (3-5), Δ^9-desaturase which is a nonheme iron protein, and a lipid micelle (6). Both oxygen and NADH are required as cofactors in this reconstituted system.

It is also reported that in intact microsomal membranes, NADPH provides the electrons to Δ^9-desaturase via another electron-transport chain which includes the flavoprotein, NADPH-cytochrome c reductase (F_{PT}) instead of

[1]Abbreviations used: F_{PT}, NADPH-cytochrome c (P-450) reductase; cyt. b_5, cytochrome b_5; anti-F_{PT}, antibody against the hydrophilic part of NADPH-cytochrome c (P-450) reductase; anti-cyt. b_5, antibody against the hydrophilic part of cytochrome b_5; γ-IgG, γ-immuno globulin fraction.

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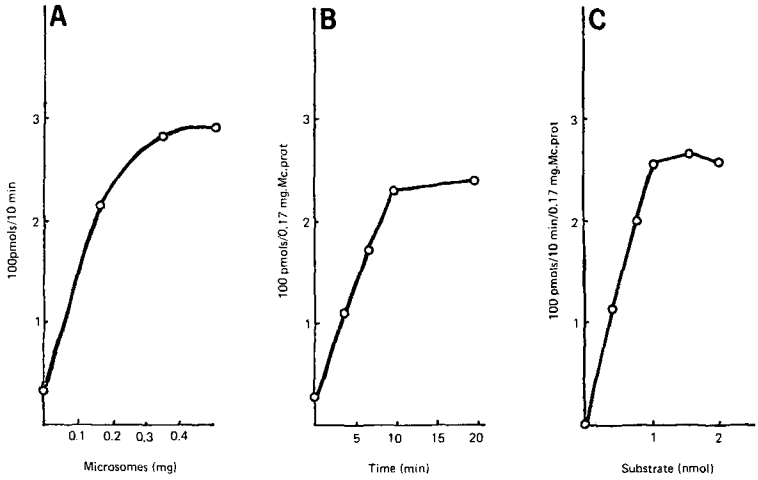

FIG. 1. Dependence of Δ^6-desaturase activity in γ-linolenic acid formation on microsomal protein concentration (A), time of incubation (B), and substract concentration (C). Incubations were carried out for 10 min (A,C) in the presence of 0.17 mg microsomal protein (B,C) and of 1 nmol of [1-^{14}C]-linoleic acid (A,B) as described in the text. 0.87 mM NADH was added to initiate the reaction.

TABLE I

Pyridine Nucleotides Requirement and Cyanide Effects on the Δ^6-desaturase Activity Converting Linoleic Acid to γ-Linolenic Acid

Nucleotides[a]	Cyanide	Δ^6-Desaturase activity γ-Linolenic acid formed	
	mM	nmol/10 min/mg.Mc.prot.[b]	(%)
	–	1.31	(100)
NADH	1	0.79	60
	5	0.24	18
	–	1.09	(100)
NADPH	1	0.87	80
	5	0.43	39

[a]NADH = reduced nicotinamide adenine dinucleotide, NADPH = reduced nicotinamide adenine dinucleotide phosphate.

[b]mg.Mc.prot., mg of microsomal protein

NADH-cytochrome b_5 reductase, and cyt. b_5 (3). The same cofactor requirement, as was found in Δ^9-desaturation by microsomal membranes, has been shown in the Δ^6-desaturation which catalyzes linoleic acid to γ-linolenic acid conversion (7). Therefore, the effects of antibodies against cyt. b_5 and F_{PT} on Δ^6-desaturase have been tested to see whether the same electron transport components in microsomal membranes are involved in both Δ^9- and Δ^6-desaturation systems.

Evidence for the participation of cyt. b_5 in the Δ^6-desaturation reaction has been obtained from immunological experiments.

MATERIALS AND METHODS

Chemicals

(1-^{14}C)-Linoleic acid (56 mCi/mmol) was provided by The Radiochemical Centre, Amersham, England. Adenosine triphosphate (ATP), coenzyme A (CoASH), NADH, NADPH, and glutathione were purchased from Kyowa Hakko Co., Japan.

Preparations of Microsomes and Antibodies

Male Wistar strain rats weighing 100 to 120 g were fed ad libitum with "Oriental" brand feed and killed by decapitation. This method of preparing untreated rat liver microsomes is similar to that of Catalá et al. (8). The microsomal pellet was suspended in the homogenizing medium to obtain a concentration of 17 mg/ml. The hydrophilic parts of F_{PT} and cyt. b_5 were purified from untreated rat liver microsomes according to the method of Omura and Takesue (9) with trypsin digestion followed by Sephadex G-100 gel filtration and DEAE-cellulose column chromatography. The specific activity and specific content of the hydrophilic portions of F_{PT} and cyt b_5 were 38 nmoles cytochrome c reduced/min/mg of protein and 70 nmoles/mg of protein, respectively. Adult male rabbits were immunized with either hydrophilic part of cty. b_5 by the method of Oshino and Omura (3) or with hydrophilic part of F_{PT} by the method of Pederson et al. (10). Blood was collected from ear veins and serum was separated from the whole blood. The DEAE-cellulose fraction of γ-globulin (γ-IgG) from both immune and preimmune serum was prepared by the procedure of Masters et al. (11).

NADH- and NADPH-Cytochrome c Reductase Assays

The assays were carried out with a Hitachi 124 spectrophotometer. In order to determine reductase activity, the microsomes were added to a 0.1 M potassium phosphate buffer (pH 7.5), which contained 0.02 μmol of cytochrome c (Boeringer, horse heart), 1 μmol of KCN, and 0.1 μmol of either NADH or NADPH. The final volume was 1.0 ml. Reduced pyridine nucleotide was added to initiate the reaction.

Δ^6-Desaturase Assay

A reaction mixture containing 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 CoASH, 0.33 mM nicotinamide, 5 mM $MgCl_2$ 0.87 mM NADH or NADPH, 1 nmol of [1-^{14}C]-linoleic acid (120,000 cpm) dissolved in propylene glycol, and microsomes (0.17 mg of protein) in a final volume of 3.2 ml was incubated for 10 min at 35 C (8). Reactions were stopped by the addition of 2 ml of 10% potassium hydroxide in

methanol. Fatty acids were extracted three times with 2 ml of diethyl ether after saponification for 45 min at 80 C under nitrogen and acidified with 1 N HCl. Fatty acids were methylated with diazomethane. The resulting methyl esters were separated according to the degree of unsaturation on thin layer plates impregnated with 4% $AgNO_3$. The developing phase used was n-hexane-ether-acetic acid (94:4:2, v/v/v) (12). A further identification of products was achieved by using a Shimazu GC-4APTE gas chromatograph equipped with a combustion furnace and a radioisotope detector RID-2E, and a 1 m steel column packed with 10% EGSSX on 60-80 mesh chromosorb W treated with dimethyl-dichlorosilane. The temperature of the column was 142 C and the carrier gas flow rate was 50 ml/min. Calculation of desaturase activity was made according to the method of Raju (13). The Δ^6-desaturase activity is expressed as nmol of γ-linolenic acid formed/10 min/mg of protein.

Protein was measured by the procedure of Lowry et al. (14).

RESULTS AND DISCUSSION

Rat liver microsomal membrane desaturated linoleic acid to γ-linolenic acid in the presence of ATP, CoASH and NADH (Fig. 1, Table I). The main product of this reaction was the corresponding γ-linolenic acid (Fig. 2). The reaction of desaturation was dependent upon the concentration of microsomal protein (Fig. 1A) and was linear up to 10 min (Fig 1B). A substrate concentration of 0.31 μM was found to give maximum activity (Fig. 1C). Under optimal conditions, the specific activity of linoleic acid Δ^6-desaturase was 1.1 to 1.3 nmol γ-linolenic acid formed/10 min/mg of microsomal protein, which is similar to that reported by Peluffo et al. (15). The pyridine nucleotide requirement, and the cyanide effect on the activity of Δ^6-dasaturase are given in Table I. Both reduced pyridine nucleotides supported the Δ^6-desaturase activities to the same degree. The inhibitory effect of cyanide on Δ^6-desaturase activity are similar to that reported by Brenner et al. (16).

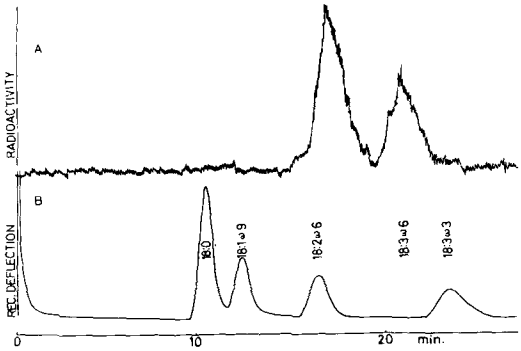

FIG. 2. Separation of fatty acid methyl esters by gas chromatography. A: Radiogaschromatogram of the incubation products. 1 nmol of [1-^{14}C]-linoleic acid was incubated for 10 min in the presence of 0.17 mg microsomal protein and 0.87 mM NADH under the conditions described in the text. B: Separation of standard fatty acid methyl esters.

In order to investigate the effects of anti-

TABLE II

Effects of Antibodies against the Hydrophilic Part of Cytochrome b_5 and NADPH-cytochrome c Reductase on the Microsomal NADH- and NADPH-cytochrome c Reductase Activity[a]

Additions of γ-IgG	NADH-cytochrome c Reductase activity		NADPH-cytochrome c Reductase activity
ratios of γ-IgG	(mg/mg.Mc.prot.)	%	%
	–	(100)	(100)
	0.45	102	99
	1.20	106	98
preimmune:	1.80	108	99
	5.5	109	99
	11.0	110	99
	0.22	101	65
immune : antibody	0.45	105	23
to NADPH-cytochrome	0.90	104	14
c reductase	1.80	106	8
immune : antibody	5.5	83	103
to cytochrome b_5	11.0	61	101

[a]NADH = reduced nicotinamide adenine dinucleotide, NADPH = reduced nicotinamide adenine dinucleotide phosphate.

TABLE III

Effects of Antibodies on the Δ^6-Desaturase Activity Converting Linoleic Acid to γ-Linolenic Acid

Nucleotide[a]	Additions of γ-IgG		Δ^6-Desaturase activity	
	ratios of γ-IgG (mg/mg.Mc.prot.)		γ-Linolenic acid formed (nmol/10 min/mg.Mc.prot.)	(%)
NADH	preimmune:	–	1.31	(100)
		10	1.48	113
		20	1.45	111
	immune : antibody to cytochrome b_5	5	1.24	95
		10	1.13	86
		20	0.90	69
		40	0.62	47
	immune : antibody to NADPH-cyt.c reductase	55	1.21	92
NADPH	preimmune :	–	1.09	(100)
		80	0.85	78
	immune : antibody to cytochrome b_5	40	0.45	41
		80	0.41	35
	immune : antibody to NADPH-cyt.c reductase	55	1.06	97

[a]NADH = reduced nicotinamide adenine dinucleotide, NADPH = reduced nicotinamide adenine dinucleotide phosphate.

bodies against cyt. b_5 and F_{PT} on the Δ^6-desaturation in microsomes, we prepared antibodies against the hydrophilic portions of cyt. b_5 (anti-cyt b_5) and F_{PT} (anti-F_{PT}) taken from rabbit serum. Both antibodies showed a single precipitin line to the microsomes solubilized with deoxycholate. The two precipitins formed between either anti-cyt. b_5 γ-IgG or anti-F_{PT} γ-IgG and microsomes were cross-reacted by the Ouchterlony diffusion method [Part of these results was reported to Biochim. Biophys. Acta (17)]. Activities of NADH-cytochrome c reductase and F_{PT} in microsomes were measured immediately after preincubation of microsomal membranes with preimmune γ-IgG, anti-cyt. b_5 γ-IgG, or anti-F_{PT} γ-IgG for 10 min at room temperature. As shown in Table II, while F_{PT} activity in microsomes was unaffected by preimmune γ-IgG, a significant decrease of NADPH-cytochrome c reductase activity was observed by the addition of antibody against the hydrophilic part of F_{PT}. Inhibition was also observed to a similar degree for NADH-cytochrome c reductase activity by antibody against the hydrophilic part cyt. b_5. On the basis of earlier studies (3), the inhibitory effects of immune γ-IgG are considered to be specific for those which required the participation of F_{PT} and cyt. b_5. The hydrophilic parts of F_{PT} and cyt. b_5 are primarily responsible for the interactions of these molecules with other redox components in the microsomal membranes.

The effect of antibodies on the Δ^6-desaturation activities are shown in Table III. Since a drastic reduction of Δ^6-desaturase activities is observed when microsomal membranes alone are preincubated for 5 min, the effects of antibodies were tested without preincubation. While the Δ^6-desaturase activity was unaffected by preimmune γ-IgG and anti-F_{PT} γ-IgG, a significant decrease of the Δ^6-desaturase activity was observed by the addition of anti-cyt. b_5 γ-IgG. These results showed a similar reduction in the Δ^6-desaturase activities when either NADH or NADPH initiated the reaction. Thus, the decrase in the activity of Δ^6-desaturase by anti-cyt. b_5 γ-IgG can be explained by the involvement of cyt. b_5 in the multi-enzyme complex of Δ^6-desaturase.

There remain some questions as to the pathway of cyt. b_5 reduction in the NADPH dependent Δ^6-desaturation. Although 92% of NADPH-cytochrome c reductase activity of intact microsomes was inhibited by anti-F_{PT} (1.8 mg/mg.Mc.prot.) the Δ^6-desaturation was not inhibited by anti-F_{PT} (55 mg/mg.Mc.prot.). When we tested the effect of anti-F_{PT} on the NADPH-dependent cyt. b_5 redox reaction in microsomes, anti-F_{PT} (4.5 mg/mg.Mc.prot.) inhibited cyt. b_5 reduction in microsomes, and the initial velocity and the steady state level became the half value of the control (unpublished results). The above facts indicate that F_{PT} transfers the reducing equivalents for the Δ^6-desaturation reaction from NADPH to cyt. b_5. When anti-F_{PT} was added to the Δ^6-

desaturation system, Δ^6-desaturation could take place, because full reduction of cty. b_5 is not necessary for the desaturations. It was reported that protease-treated microsomes, containing only about 10% of the original cyt. b_5, exhibited the same Δ^9-desaturation activity as was found in intact microsomes (3). At the same time, it was shown that saturated palmitic and stearic acids in amounts equivalent to the substrate did not inhibit linoleic acid desaturation (1). Recently, Enoch et al. (18) also reported that hydrogen removal is the rate limiting step of stearyl-CoA desaturation. It is possible that the same electron carrier, that is cyt. b_5, participates in both Δ^6- and Δ^9-desaturation systems in microsomal membranes.

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Glycerokinase in Human Adipose Tissue

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ABSTRACT

The presence of glycerokinase has been demonstrated in human omental and subcutaneous adipose tissue. The enzyme reaction showed a linear time course for 5 min at 30 C and pH optima at pH 7.6 and 9.0. Saturation of the enzyme was observed at 1.8 mM adenosine triphosphate (ATP) and the double reciprocal plot of activity vs. ATP concentration was nonlinear giving two apparent Km values of 0.094 and 0.518 mM. The apparent Km for glycerol, 0.112 mM, was obtained from a linear double reciprocal plot, and the enzyme was saturated at about 0.4 mM glycerol. The activity of glycerokinase in human adipose tissue excised under general anaesthesia was low and was unrelated to adipose cell size or the degree of obesity of the subject from whom the fat was obtained.

INTRODUCTION

The development of a radiochemical assay (1) established the presence of glycerokinase (adenosine triphosphate:glycerol phosphotransferase, EC 2.7.1.30) in rat adipose tissue (2) and raised the possibility that the enzyme might be involved in the development of obesity (3). Further studies have confirmed the presence of glycerokinase in rats (4,5) and have also shown that this enzyme occurs in adipose tissue from mice (6,7), domestic chickens (8), and pigs (9), and in human subcutaneous adipose tissue (10). The objectives of the present investigation were to establish optimal conditions for assaying glycerokinase in human adipose tissue and to determine whether any relationship exists between the activity of the enzyme and adipose cell size. Since subcutaneous fat cells are almost invariably larger than omental fat cells (11), paried comparisons were performed on omental and subcutaneous adipose tissue obtained from each of a series of subjects undergoing elective abdominal surgery.

MATERIALS AND METHODS

Source of Adipose Tissue

Adipose tissue was obtained from subjects undergoing elective cholecystectomy or hysterectomy. Operations were performed in the mornings following an overnight fast. Morphine and atropine or their derivatives were used for premedication. Anaesthesia was induced with thiopentone sodium and maintained with nitrous oxide and halothane. Tissues were transported to the laboratory in 0.15 M sodium chloride at 37 C and processed within 15 min. Preliminary studies to define optimal conditions for assaying glycerokinase were performed on adipose tissue from the greater omentum. Comparative studies were performed on fat obtained from the subcutaneous tissues of the anterior abdominal wall and the greater omentum of 18 subjects. Relevant clinical data on these 18 subjects as well as individual values for adipose cell size are shown in Table I. Most of the subjects were females. Their body weights standardized for age, sex, and height (12) averaged 18% in excess of ideal (range-6% to + 37%). Paired comparisons of adipose cell size (expressed as μg triolein per cell) showed that subcutaneous fat cells were 73% larger ($P<0.001$) than omental fat cells.

Assay of Glycerokinase

Adipose tissue was homogenized for 2 min at 0 C in 2 volumes of 2% KCl in 1 mM EDTA. The homogenate was centrifuged at 17,000 x g for 10 min at 4 C. The aqueous layer was centrifuged at 100,000 x g for 30 min at 4 C. The supernate was used directly in the assay which was performed using a modification of the procedure of Newsholme et al. (1). The reaction mixture contained the following components in a final volume of 130 μl: 0.04 μmoles glycerol -^{14}C (specific activity 16.2 μCi/μmole); 0.44 μmoles adenosine triphosphate (ATP); 1.3 μmoles ethylene diamine tetracetic acid (EDTA); 3.25 μmoles NaF; 13 μmoles tris-HCl, pH 7.6. The reaction was started by the addition of 50 μl of homogenate and allowed to proceed for 5 min at 30 C. The reaction was stopped by the addition of 100 μl absolute ethanol. Controls consisted of reaction mixtures to which ethanol was added before the enzyme extract. The protein was removed by centrifugation, and 20 μl of the deproteinized reaction mixture were applied transversely at one end of a 1.5 x 6 cm strip of Whatman DE 81 chromatography paper. Glycerophosphate was separated from glycerol by descending

TABLE I

Clinical Data

Subject	Age (yr)	Sex	% IBW[a]	μg triolein per cell Adipose tissue	
				Omental	Subcutaneous
1	19	F	125	0.235	0.546
2	25	F	104	0.208	0.590
3	55	M	132	0.880	1.285
4	35	F	137	0.439	0.766
5	63	F	135	0.238	0.565
6	23	F	115	0.264	0.485
7	57	M	117	0.746	0.720
8	49	F	104	0.333	0.890
9	35	F	133	0.259	0.710
10	77	M	94	0.712	0.590
11	36	M	117	0.292	0.482
12	23	F	130	0.140	0.361
13	61	F	121	0.438	0.918
14	45	F	106	0.364	0.524
15	27	F	113	0.334	0.593
16	44	F	100	0.173	0.413
17	65	F	133	0.271	0.312
18	32	F	106	0.275	0.656
Means ± SEM	43.8 ± 4.0		118 ± 3	0.367 ± 0.049	0.634 ± 0.054

[a]Percentage of ideal body weight

chromatography in distilled water. Glycerophosphate was recovered in the area closest to the origin; glycerol ran with the solvent front. The papers were dried in air and their radioactivity was measured in a liquid scintillation system using a toluene scintillator (0.05 g dimethyl POPOP and 4 g PPO per liter toluene). The product of the assay had the same Rf values as glycerophosphate when tested in the present separation system and when subjected to descending chromatography on Whatman No. I paper in the following solvent systems (13): (a) ethyl acetate:acetic acid:water = 3:3:1, (b) methyl cellosolve:methyl ethyl ketone:3M NH_4OH = 7:2:3. The amount of product formed was calculated from the specific activity of glycerol in the assay system and the radioactivity measured in glycerophosphate. The data have been expressed as n moles glycerol converted per minute either per mg protein or per 10^6 adipose cells. The coefficient of variation of replicate analyses of a single homogenate was 4.9%.

Analytical Methods

Protein was estimated by the method of Lowry et al. (14). Glycerol was measured enzymatically (15) in homogenates which had been deproteinized with perchloric acid and neutralized with potassium bicarbonate. The conversion of reduced trinicotinamide adenine dinucleotide (NADH) to nicotinamide adenine dinucleotide (NAD) was measured in an Aminco-Bowman spectrofluorimeter using excitation and emission wavelengths of 340 and 460 nm, respectively. The assay system was adapted for fluorimetry by reducing the concentration of NADH to 100 nmoles in a final volume of 1 ml. The concentration of glycerol was estimated from internal standards spanning the range 5-60 nmoles. The diameter of adipose cells was measured (16) on at least 500 isolated fat cells prepared (17) from a subsample of the specimen of adipose tissue and is expressed as μg triolein per cell. The latter was calculated from the mean cell volume (18) and the specific gravity of triolein. The number of fat cells in the sample of adipose tissue used in the glycerokinase assay was derived from the wet weight of the tissue as follows. A subsample of adipose tissue was weighed and then extracted for 48 hr at 4 C in isopropyl alcohol:heptane: 0.1 M sulphuric acid 4:1:0.1 v/v. The extract was converted to a two phase system (19) and the triglyceride content was measured (20) on an aliquot of the upper phase. The number of adipose cells in a given wet weight of tissue was calculated by dividing the triglyceride content by the weight of triolein per cell. Paired comparisons of the data were performed by the analysis of vairance and correlation coefficients were computed by covariance analysis (21).

Materials

All enzymes and cofactors for the glycerol and glycerokinase assays were obtained from

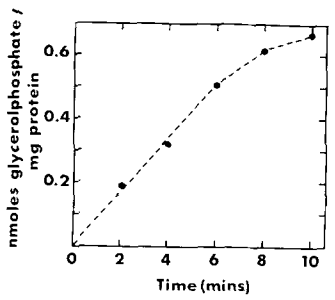

FIG. 1. Time course of glycerophosphate production at 30 C. The data represent the means of six experiments.

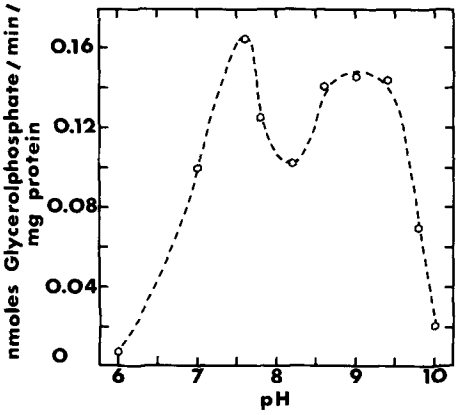

FIG. 2. Effects of pH on the production of glycerophosphate. Tris-HCl was used for all values of pH except at Ph 6.0 when tris-maleate buffer was used. The data represent the means of three experimentsl.

the Boehringer Corp. (London) Ltd. Other materials and their suppliers were as follows: collagenase (Worthington Biochemical Corp., Freehold, NJ); DL-α-(di) sodium glycerophosphate, 6 H_2O (Mann Research Laboratories, Orangeburg, NY); Whatman DE81 and Whatman No. I chromatography papers (W & R Balston Ltd., England); and glycerol-^{14}C (Radiochemical Centre, Amersham, Bucks, England). The glycerol-^{14}C was purified (>99.6%) before use by descending chromatography in distilled water on Whatman DE81 paper.

RESULTS

Preliminary studies showed that conditions for assaying glycerokinase in rat adipose tissue failed to permit a linear production of glycerophosphate withtime. This problem was not corrected by using an ATP regenerating system (2) but was overcome by carrying out the assay at 30 C. Mercaptoethanol had unpredictable effects and was therefore omitted. With the present system, the production of glycerophosphate was linear for 6 min (Fig. 1), it was 2.5-fold greater than that observed when the assay was performed at 37 C for 30 min and was proportional to the concentration of protein over the range 20-160 μg. Enzyme activity was undetectable in the absence of tissue extract and was destroyed by boiling for 5 min. Activity was also destroyed by preincubating the tissue homogenate with 10 mM iodoacetamide in 0.03 mM tris buffer, pH 7.4 for 75 min. In the latter respect, the glycerokinase in human adipose tissue exhibited similar characteristics to that in rat liver (22). As shown in Figure 2, the human enzyme exhibited pH optima at 7.6 and 9.0. For most glycerokinases, the optimum lies in the range pH 9.0-9.8 (23) although some have an optimum between pH 7.0-7.5 (24). The reason for the double peak in human adipose tissue is uncertain, but because the lower optimum is closer to physiological, a pH of 7.6 was used for all assays.

The effect of varying the concentration of ATP is shown in Figure 3. In the absence of exogenous ATP, ca. one-third of maximal activity was found and the data have been corrected for endogenous ATP. The human enzyme exhibited a downward curvature of the double reciprocal plot giving two apparent Km values of 0.094 and 0.518 mM. This unusual property appears to be characteristic of glycerokinases insofar as it has been demonstrated in rat, beef, and human liver and in *Candida mycoderma* (25) as well as in crystalline glycerokinase from *Escherichia coli* (26). The data in Figure 3 show that the enzyme was saturated at 1.8 mM ATP.

The effect of varying the concentration of glycerol is shown in Figure 4. In contrast to the findings with ATP, the double reciprocal plots of enzyme activity vs. glycerol concentration were linear with saturation occurring at about 0.4 mM. The mean ± S.E.M. apparent Km for glycerol was 0.112 ± 0.009 mM. Corresponding values for rat adipose tissue vary from 0.01 mM (2) to 1.0 mM (5). The apparent Km for glycerol with the glycerokinase in chicken adipose tissue is 0.153 mM (8) and is of the same order of magnitude as that observed in the present investigation.

Table II lists the average values for glycerokinase activity as well as concentrations of glycerol and protein measured in homogenates prepared from the tissues of subjects in Table I. When the data in Table II were expressed on a per cell basis, glycerokinase activity was similar in subcutaneous and omental adipose tissue from the same subject. The protein concentrations of the two tissues were also similar. How-

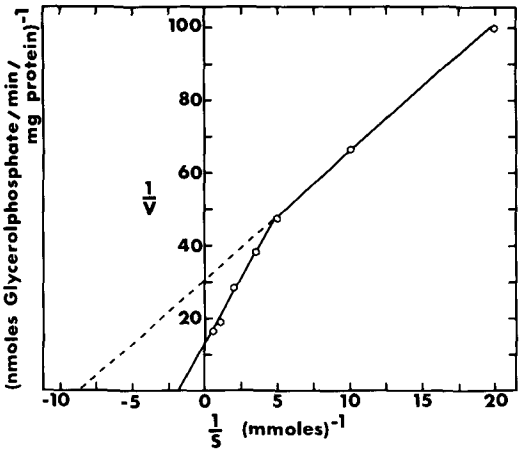

FIG. 3. Double reciprocal plot of glycerophosphate production versus the concentration of adenosine triphosphate (ATP). The data represent the means of four experiments.

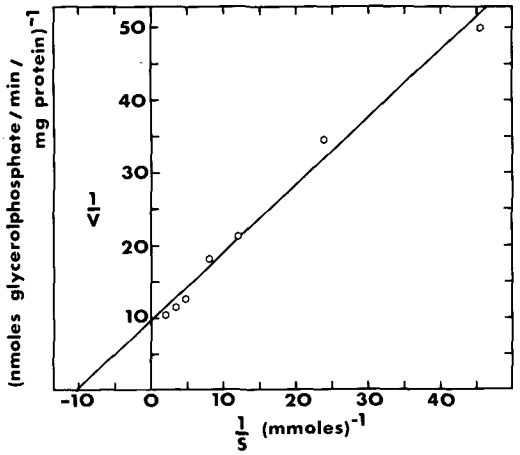

FIG. 4. Double reciprocal plot of glycerophosphate production versus the concentration of glycerol. The data represent the means of seven experiments.

ever, glycerokinase activity expressed per mg protein was significantly greater in omental than subcutaneous adipose tissue. Since glycerokinase is confined to adipocytes (4,5,27,28) and the latter contribute only about 50% of the total protein in adipose tissue (29), it appears that tissue protein is a poor reference for comparing the activity of this enzyme in fat from different sites. In effect, paired analyses have failed to shown any difference in glycerokinase activity between large and small fat cells from the same subject. Furthermore, there was no relationship between glycerokinase activity and adipose cell size in subcutaneous or omental adipose tissue. There was also no relationship between glycerokinase activity and the degree of obesity. However, it should be emphasized that the range of relative body weights of these subjects was fairly small.

The data for tissue glycerol concentrations listed in Table II can be used to estimate the error involved if dilution of the labelled glycerol by tissue glycerol is not corrected for when computing the production of glycerophosphate. Using the average values for tissue glycerol/g adipose tissue, it can be calculated that glycerokinase activity will be underestimated by 10% in subcutaneous adipose tissue and by 5% in omental adipose tissue if the glycerol content of the homogenate is not taken into account. It should be emphasized, however, that these estimates apply only to the conditions of the present experiments because it is very likely that the tissue glycerol concentrations were artificially high due to lipolysis that occurred between removal of tissues at surgery and processing in the laboratory. This can be deduced from the fact that glycerol production is increased in enlarged fat cells (11) and, as shown in Table II, tissue glycerol concentrations were higher in subcutaneous than in omental adipose tissue. Furthermore, not only were subcutaneous fat cells larger than omental fat cells, but also the concentration of tissue glycerol/10^6 cells was directly proportional to adipose cell size in omental (0.701; $P<0.001$)

TABLE II

Metabolic Data on Omental and Subcutaneous Adipose Tissue[a]

	Omental	Subcutaneous	
Glycerokinase activity			
(nmoles glycerol/10^6 cells/min)	0.24 ± 0.04	0.30 ± 0.07	N.S.
(nmoles glycerol/mg protein/min)	0.15 ± 0.003	0.08 ± 0.02	$P<0.05$
Glycerol (μmoles/g tissue)	0.082 ± 0.009	0.161 ± 0.028	$P<0.01$
Glycerol (μmoles/10^6 cells)	0.042 ± 0.009	0.139 ± 0.023	$P<0.001$
Protein (mg/g tissue)	4.56 ± 0.50	5.20 ± 0.54	N.S.

[a]Values are given as means ± SEM

and subcutaneous ($r=0.592$; $P<0.01$) fat.

DISCUSSION

The present investigation has confirmed the findings of Koschinsky and Gries (10) that glycerokinase is present in human subcutaneous adipose tissue. We have also shown that this enzyme occurs in adipose tissue from the greater omentum and that its activity is similar in omental and subcutaneous fat. Modifications to established assay conditions (1,2) were necessary for measuring optimal enzyme activity in human tissues. These consisted principally of lowering the incubation temperature to 30 C, reducing the incubation time to 5 min, and omitting mercaptoethanol from the reaction system. Under these conditions, the requirements of the human enzyme for ATP were similar to those of other species, and the Km for glycerol was similar to that of the glycerokinase in chicken adipose tissue. The human enzyme, however, was distinguished from other glycerokinases by exhibiting two pH optima, one at 7.6 and the other at 9.0.

The activity of the glycerokinase in human adipose tissue was very low by comparison with that in laboratory animals. The potential ability of human adipose tissue to phosphorylate glycerol was ca. 1% of that in porcine adipose tissue (9), 2% of values in hens and mice (7,8), and ca. 20% of values in rat adipose cells (4). Differences of this order are not surprising because other parameters of metabolic activity in rodent, porcine, and avian adipose tissue are generally greater than in human adipose tissue. However, they may well have been exaggerated by the effects of general anaesthesia and starvation prior to obtaining samples of tissue at surgery (8). The activities observed in this investigation were ca. 20 times greater than those reported in subcutaneous adipose tissue from lean subjects and 1.5-fold greater than those reported in subcutaneous fat from grossly obese subjects (10). The higher enzyme activities recorded here cannot be attributed to differences in nutritional state and are at least partly accounted for by differences in assay conditions. We were unable to show any relationship between adipose cell size and glycerokinase activity nor could we confirm previous reports that glycerokinase activity is elevated in adipose tissue of obese humans (10). However, in the latter connection, it should be emphasized that the subjects studied here encompassed a fairly narrow range of relative body weights.

The physiological importance of glycerokinase in human adipose tissue remains to be defined. If the enzyme is saturated with substrate, the present findings indicate that omental adipose tissue can phosphorylate 0.24 nmoles glycerol/10^6 cells/min. Basal glycerol production in human omental fat cells averages 0.933 nmoles/10^6 cells/min (11). On this basis, the true rate of glycerol production is 1.173 nmoles/10^6 cells/min and reutilization of glycerol represents 20.5% of the total. Similar calculations show that 14.7% of glycerol produced under basal conditions by subcutaneous fat cells (11) is reutilized. These calculations assume inter alia that the intracellular concentration of free glycerol is of the order of 0.4 mM which represented a saturating concentration of glycerol in the glycerokinase assay. On the other hand, glycerol is freely diffusible and its concentration in plasma in the post absorptive state is ca. 0.05 mM (30). If the intracellular concentration of glycerol corresponds to that in plasma, it can be calculated from the Km for glycerol that glycerokinase will phosphorylate 0.08 nmoles glycerol/10^6 cells/min in omental adipose tissue or reutilize 7.9% of the glycerol produced. In the case of subcutaneous adipose tissue, glycerokinase may only phosphorylate 0.1 nmoles glycerol/10^6 cells/min which represents a 5.2% reutilization of the glycerol produced. Thus, in the final analysis, accurate data on the effects of dietary changes and obesity on the intracellular concentration of glycerol and on the activity of glycerokinase will be required before a regulatory role for glycerokinase in human adipost tissue can be defined.

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A Comparison of Simplified Methods for Lipoprotein Quantification Using the Analytic Ultracentrifuge as a Standard

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ABSTRACT

Two simplified methods for quantitative lipoprotein analysis have been calibrated and compared with each other using analytic ultracentrifugation as a standard reference procedure. The first method was the Friedewald procedure and the second was an automated agarose gel electrophoresis system. Both procedures offer comparable quantitative lipoprotein analysis with potential for large scale screening purposes at low cost ($4.00-$5.00 per analysis). There were advantages and limitations to both procedures. The Friedwald procedure can be used on frozen sera but requires 3 ml sera. In contrast, the electrophoresis system must be used with fresh serum but requires only 50 µl serum and the electrophoretic slides may be quantitatively analyzed several years retrospectively.

INTRODUCTION

During the past decade the Fredrickson, Levy, and Lees "typing" system has allowed classification of most lipoprotein disorders into six types (1). However, a major limitation of this typing system is that it does not provide quantitative lipoprotein data. Such data provide additional information about coronary heart disease (CHD) risk associated with elevated serum cholesterol (TC) and triglyceride (TG) levels. For example, a moderately elevated cholesterol (230-260 mg/100 ml) may be the result of substantial elevations of either the "atherogenic" LDL (2) or the "non-atherogenic" HDL class. Thus, the major classes of lipoproteins, very low density lipoproteins (VLDL), low density lipoproteins (LDL), and high density lipoproteins (HDL) need to be quantitatively measured in clinical laboratories. Measurement of HDL is particularly important since there is recent evidence to suggest that HDL is inversely related to CHD prevalence and that this relationship is largely independent of total cholesterol and LDL levels (2).

Presently the Lipid Research Clinics (LRCs) measure these lipoproteins by a combination of centrifugation and precipitation procedures (3) to obtain the cholesterol content of the three major lipoprotein classes. However, this method requires 5 ml of serum, and because of the centrifugation step, it is severely limited in the number of samples that can be processed.

The purpose of this study was to evaluate simplified quantitative lipoprotein procedures. A simplified $MnCl_2$-heparin precipitation analysis (4) was compared to the analytic ultracentrifuge, which, in this study, was the lipoprotein standard method. The simplified procedures require only 3.0 ml serum and omits the centrifugal step by estimating VLDL cholesterol directly from the serum value TG/5 (4). For comparison as another potentially useful clinical test, a recently automated lipoprotein electrophoresis system (5) was similarly evaluated.

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TABLE I

Comparison of Analytic Ultracentrifuge Lipoprotein Results with Data from a Modesto Normal Reference Population (6)[a]

Population	HDL	VLDL	LDL
This study (n = 37) age 20-39 yrs	357 ± 86	57 ± 50	354 ± 109
Modesto normals (n = 28) age 25-39 yrs	339 ± 58	43 ± 30	319 ± 74

[a]HDL = high density lipoprotein, VLDL = very low density lipoprotein, LDL = low density lipoprotein.

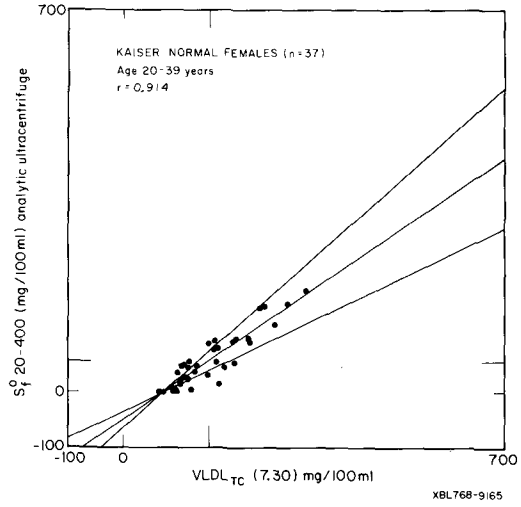

FIG. 1. Comparison of Friedewald very low density lipoprotein (VLDL) data with S_f^0 20-400 analytic ultracentrifuge data.

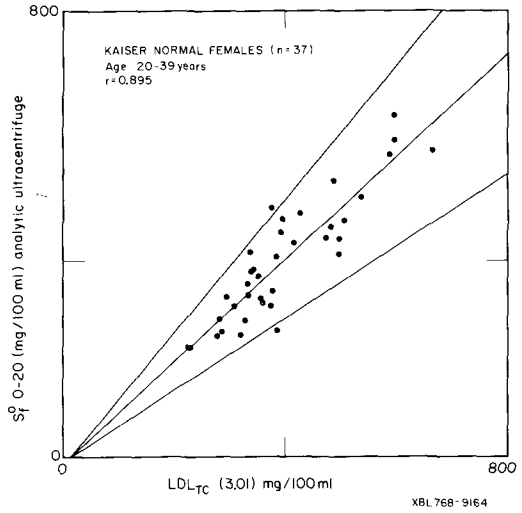

FIG. 2. Comparison of Friedewald low density lipoprotein (LDL) data with S_f^0 0-20 analytic ultracentrifuge results.

METHODS

Lipoprotein analysis was done on serum obtained from 39 fasting volunteer women, aged 20-39 yr, who had multiphasic tests as part of a routine medical examination at the Kaiser Hospital, Walnut Creek, CA (6). Normal, free-living women were selected as subjects since they exhibit a greater range in serum HDL concentration than do men of the same age group (7).

The first method used was the Friedwald procedure (4), as described in detail elsewhere (3). Briefly, 120 µl of heparin solution (5,000 I.U./ml, Riker Laboratories, Northridge, CA) is added to 3 ml serum. After vortex mixing, 150 µl of 1.0M $MnCl_2$ solution is added and again vortexed. After 30 min at 0 C, the VLDL and LDL precipitate was centrifuged at 0 C for 30 min at 1,500 x g. Then a filtrate (Lipo-Frax, Technicraft, San Mateo, CA) was made yielding the HDL containing serum solution for cholesterol analysis. Triglyceride and cholesterol analyses were made by the Technicon AA II procedure (8,9), with satisfactory phase 2 standardization by the Control Disease Center, Atlanta, GA. Lipoprotein concentrations were calculated assuming a mean wt% cholesterol content for VLDL, LDL, and HDL of 14%, 33% and 17%, respectively (10).

Lipoprotein electrophoresis was performed using the Bio-Gram A Lipoprotein Profile Kit (Bio-Rad Laboratories, Richmond, CA) with modifications described earlier (10). Results were internally standardized using both serum triglyceride and cholesterol measurements and

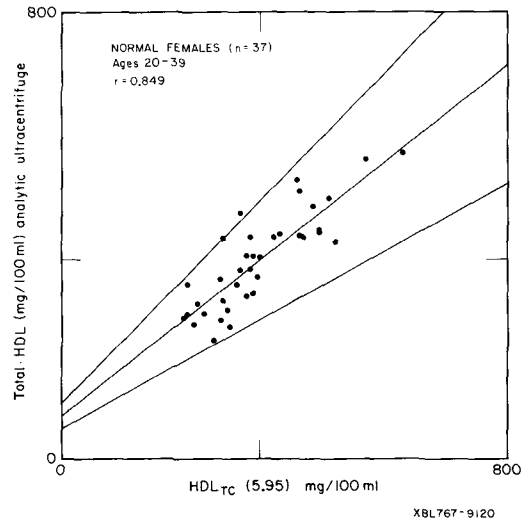

FIG. 3. Comparison of Friedewald high density lipoprotein (HDL) data with $F_{1.20}$ 0-9 analytic ultracentrifuge data.

were independent of the amount of sample (0.75-1.50 µl) applied to the gel. VLDL, LDL, and HDL concentrations were calculated from the relative dye uptake factors, the mean wt% content of TG and TC in each lipoprotein class, and the serum total TG and TC values. The hardware consisted of a densitometer, an analog to digital converter, a cathode ray tube terminal (with connection to a large computer), a teleprinter and a small computer. Full details of this automated microdensitometry facility have

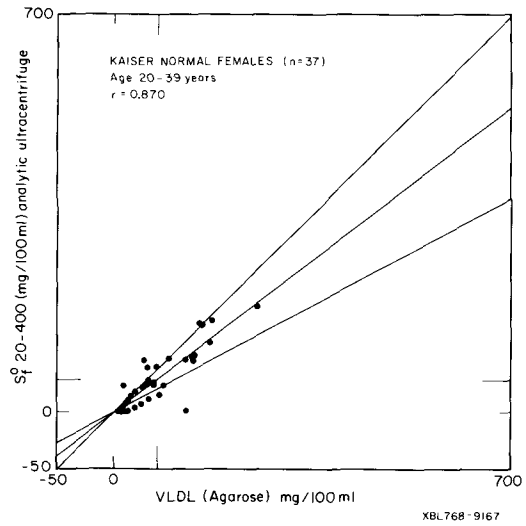

FIG. 4. Comparison of agarose gel electrophoretic pre-beta very low density lipoprotein (VLDL) data with S_f^0 20-400 analytic ultracentrifuge results.

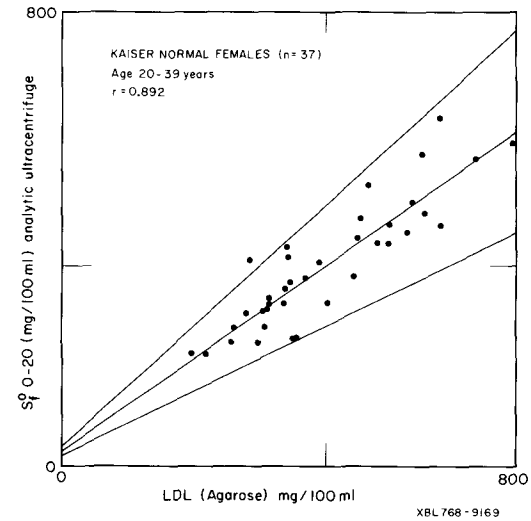

FIG. 5. Comparison of agarose gel electrophroretic beta low density lipoprotein (LDL) data with S_f^0 0-20 analytic ultracentrifuge results.

been given elsewhere (5). Both of the above simplified procedures were compared and calibrated using analytic ultracentrifugation (11) as a standard reference procedure.

RESULTS

Quantitative lipoprotein determinations were obtained using each of the three procedures: complete analytic ultracentrifugation, quantitative agarose gel electrophoresis, and by

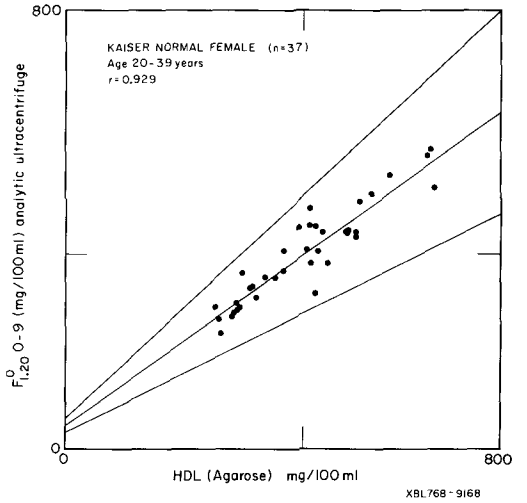

FIG. 6. Comparison of agarose gel electrophoretic alpha high density lipoprotein (HDL) data with $F_{1.20}^0$ 0-9 analytic ultracentrifuge data.

the Friedewald $MnCl_2$-heparin precipitation procedure. Analytic ultracentrifuge results, although somewhat lower for LDL in this study, are given in Table I and were not significantly different from a normal reference population studied earlier (7).

Comparison of the Friedewald-$MnCl_2$-heparin procedure with analytic ultracentrifugation is given in Figures 1, 2, and 3. Similar comparison of automated agarose electrophoresis is given in Figures 4, 5, and 6. Correlation coefficients for all data were 0.849, 0.914, and 0.895 for HDL, VLDL, and LDL, respectively, for the Friedewald proceudre. Analogous correlation for electrophoresis were 0.929, 0.870, and 0.892, respectively. Thus, comparable quantitative lipoprotein data may be expected from these two simplified procedures. Table II gives a more detailed comparison of the two methods, including regression formulae for both procedures allowing calculation of equivalent ultracentrifuge data. Also, comparison of the error of measurement in estimating the assumed true value, i.e., the analytic ultracentrifuge results, is given by Sy.x. Although agarose provides a somewhat better estimation for HDL, no definitive advantage in accuracy is provided by either method.

Evaluation of the frozen standard by the electrophoretic procedure is possible only for a standard very low in VLDL (10). For such a standard over a period of 1 yr with 24 analyses, the mean and SD values for LDL and HDL were 420 ± 33 and 301 ± 24, respectively. In contrast, no such restrictions are involved in a frozen standard for the Friedewald procedure.

TABLE II

Comparison of Electrophoretic and $MnCl_2$-Heparin Procedures with Analytic Ultracentrifugation[a]

Variable	Mean ± SD	r	$S_{y \cdot x}$	b	a
Agarose HDL	438 ± 111	0.929	33	43	0.717
Agarose VLDL	77 ± 57	0.870	26	2	0.770
Agarose LDL	466 ± 138	0.892	51	27	0.702
$MnCl_2$-Hep. HDL[b]	355 ± 95	0.849	47	78	0.785
$MnCl_2$-Hep. VLDL[c]	158 ± 67	0.914	21	−51	0.686
$MnCl_2$-Hep. LDL[d]	398 ± 106	0.895	50	−21	0.920

[a]y = b + ax, where y = Analytic Ultracentrifuge Lipoprotein Values. HDL = high density lipoprotein, VLDL = very low density lipoprotein, LDL = low density lipoprotein.

[b]HDL = 5.95 x HDL_{TC}.

[c]VLDL = Serum TG/5 x 7.30.

[d]LDL = (Serum TC - HDL_{TC} - TG/5) 3.01.

A typical standard analyzed 36 times over a 9 day period gave 304 ± 16, 149 ± 14, and 341 ± 48 for HDL, VLDL, and LDL, respectively.

DISCUSSION

Two methods for quantitative lipoprotein analysis have been calibrated and compared. Each procedure required 50 μl-3 ml of serum, in addition to that needed for serum triglyceride and cholesterol analysis. Both methods gave comparable accuracy and reproducibility for VLDL, LDL, and HDL. Advantages of the Friedewald type procedure were the capability for analyzing frozen serum samples after prolonged storage as well as a single precipitation step for all samples with TG < 400 mg/100 ml. The disadvantage of this procedure was the necessity of performing accurate cholesterol analyses on HDL containing solutions whose cholesterol concentrations were a fraction of the total serum value.

The automated lipoprotein electrophoresis also provided satisfactory quantitative data for VLDL, LDL, and HDL. Two advantages of this method are the capability of analysis on 50 μl or less of serum if enzymatic TG and TC lipid analyses are performed (12). Secondly, the agarose slides are stable for several years, allowing convenient analysis (or re-analysis) at any future time.

Perhaps the main alternatives to the above procedures would be the quantitative lipoprotein measurement now utilized by the Lipid Research Clinics (3). However, only limited numbers of samples can be done by this centrifugation and $MnCl_2$-heparin precipitation procedure. One technician, with the needed preparative ultracentrifuge, can handle only some 36 analyses per week. The estimated cost per complete analysis is $10.00. Furthermore, this procedure requires 5 ml of serum exclusive of that needed for the total serum TG and TC measurements. On the other hand, using the Friedewald procedure and requiring 3 ml serum, one technician can process 85 samples per week with an approximate cost of $4.00 per analysis (exclusive of the serum TC and TG determinations). By comparison, one technician using the automated electrophoresis system, and requiring as little as 50 μl of serum, can process ca. 250 samples per week (exclusive of the serum TC and TG determinations). A rough estimate of this cost (also exclusive of the lipid determinations) is ca. $5.00 per analysis. Considering the above, the two simplified procedures would appear potentially to offer distinct advantages over the LRC procedure in cost, serum volume requirements, and large scale screening capability.

ACKNOWLEDGMENTS

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Composition of Novel Triesters from the Skin of the Rhino Mutant Mouse[1]

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ABSTRACT

Composition of two novel triesters, derived from the skin of the rhino mutant mouse, is described. Chemical and spectroscopic analysis of the products of pancreatic hydrolysis of the triesters showed that these are comprised predominantly of isomer I (92.7 mole %). The syntheses of two reference compounds, 1-O-hexadecanoyl-2-[(14-hexadecanoyloxy)O-tetradecanoyl] 1,2-hexadecanediol (Ia) and 2-O-hexadecanoyl-1-[(14-hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (IIa), corresponding in their structures to isomers I and II of the triester, wax have also been described.

INTRODUCTION

In a previous communication (1), we have reported the occurrence of triesters, a novel neutral lipid, in the skin of the rhino mutant mouse. This lipid was shown to be composed of three structural units involving fatty acids, ω-hydroxy fatty acids and long chain 1,2-alkanediols. Two alternate structures, 1-O-acyl-2-[(ω-acyloxy)O-acyl]-1,2-alkanediol (I) or 1-[(ω-acyloxy)O-acyl]-2-O-acyl-1,2-alkanediol (II), were proposed for this lipid. In the present report, we describe a further distinction between these two structures on the basis of spectroscopic and chemical evidence.

MATERIALS AND METHODS

For this study, male and female homozygous rhino ($hr^{rh}hr^{rh}$) mice on a C57BL10J-related background, 5 mo old or older, were obtained from the Skin and Cancer Hospital Animal Colony. They were fed on a commercial breeder diet ad libitum.

Chromatographic procedures and the method used for the isolation of the triesters were the same as described previously (1).

Nuclear magnetic resonance (NMR) spectra were recorded at 220 MHz on Varian Hr-220 NMR spectrometer in 0.4-0.5 ml distilled carbon tetrachloride using tetramethylsilane as internal standard. Analyses were run at about 25 C on 10-15 μmol samples using Fourier transform. Infrared (IR) spectra were determined as solid or liquid film on AgCl plates on a Perkin-Elmer 237-B spectrophotometer.

CrO_3-pyridine complex (sarett reagent) was prepared by the procedure of Ratcliffe and Rodehorst (2). Jones reagent (chromic acid/acetone) was prepared by a modified procedure of Djerassi et al. (3). Silylation was done with pyridine:hexamethyldisilazane:trimethylchlorosilane 9:3:1. Alkaline hydrolysis was performed as described in (4). 1,2-Hexadecanediol was prepared by lithium aluminum hydride reduction of methyl 2-hydroxy-hexadecanoate. Hexadecanoic anhydride was prepared according to Selinger and Lapidot (5). 1,14-Tetradecanediol was purchased from Eastman Kodak, Rochester, NY.

Preparation of Ia and IIa (Fig. 1)

To distinguish between isomers I and II of triester wax, two model compounds, 1-O-hexadecanoyl-2-[(14-hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (Ia) and 2-O-hexadecanoyl-1-[(14]hexadecanoyloxy)O-tetradecanoyl]-1,2-hexadecanediol (IIa), were prepared by the following procedure. 1- or 2-O-Hexadecanoyl-1,2-hexadecanediol (IIIa or IVa,

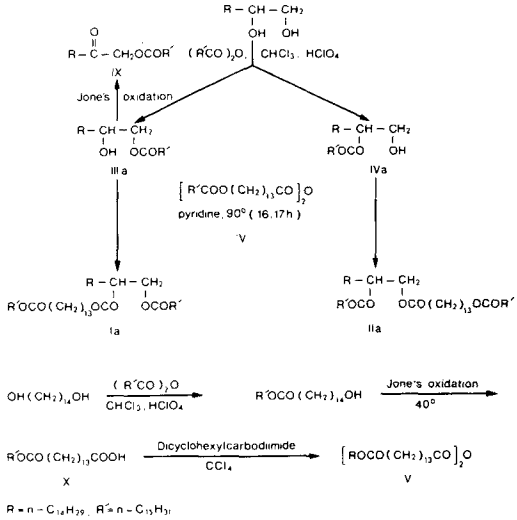

FIG. 1. Preparation of reference triester lipids (Ia and IIa) and of the intermediate materials IIIa, IVa and V.

[1]Presented at the AOCS 49th Annual Fall Meeting, Cincinnati, Sept. 1975.

[2]Author to whom correspondence should be addressed.

TABLE I

Nuclear Magnetic Resonance Spectra of the Products Obtained by Lipase Hydrolysis of the Triesters and Reference Compounds

Proton	(Multiplicity, J, Hz)					
	I or II, Ia and IIa	IVa	IV	VI	VII	VIII
CH_3	0.87 (t)	0.89 (t)	0.87 (t)	0.85 (t)	0.86 (t)	0.83 (t)
$(CH_2)_n$	1.25 (s)	1.26 (s)	1.25 (s)	1.25 (s)	1.25 (s)	1.25 (s)
Allylic methylenes	1.96 (m)[a]		1.93 (m)	1.91 (m)	1.91 (m)	1.92 (m)
alpha-methylenes	2.2 (q)[b]	2.2 (t)	2.2 (t)	2.18 (q)[b]	2.18 (t)	2.11 & 2.19[d] (t)
Position-1-methylene protons of glycol moiety	3.85 & 4.15 (dd, 6.5, 3; 11)[c]	3.43 & 3.52 (dd, 6, 3; 12)[c]	3.43 & 3.52 (dd, 6, 3; 12)[c]	3.46 & 3.54 (dd, 6, 3; 12)[c]		
Position-2-methylene protons of glycol moiety	4.93 (m)	4.76 (m)	4.77 (m)	4.77 (m)		
Methylene protons adjacent to acyloxy group	3.95 (t)			3.94 (t)		3.94 (t)
olefinic protons	5.25 (m)[a]		5.25 (m)	5.25 (m)	5.25 (m)	5.25 (m)
$COOCH_3$					3.58 (s)	3.58 (s)

[a]These signals were not present in the spectra of Ia and IIa.

[b]Appearance of alpha-methylene protons as a quartet apparently results from two partially overlapping triplets.

[c]Two nonequivalent methylene protons of glycol moiety showed different vicinal coupling constants.

[d]Two triplets were observed for methylene protons adjacent to ester carbonyl. Downfield triplet was assigned to methylene protons adjacent to carbomethoxy group by comparison with VII.

49.6 mg, 0.1 mmol was treated with 14-hexadecanoyloxy-tetradecanoic anhydride (V, 88.2 mg, 0.1 mmol) in pyridine (4 ml) at 90 C for 16-17 hr. The reaction mixture was poured over ice and extracted with ether. Combined ether extracts were dried over anhydrous sodium sulfate. Triester lipid (Ia or IIa, 96 mg, yield = 70%) was separated from 14-hexadecanoyloxy-tetradecanoic acid (X) by thin layer chromatography (TLC) (hexane/ether 95:5). NMR (Table I) and IR spectra of both isomers were identical to each other and to that of naturally occurring triester wax (6) except that signals for olefinic and allylic protons were not present in the former spectra. Alkaline hydrolysis of Ia or IIa gave hexadecanediol, 14-hydroxy-tetradecanoic acid, and hexadecanoic acid in the expected proportions.

Preparation of 1-O-Hexadecanoyl-1,2-Hexadecanediol (IIIa), and 2-O-Hexadecanoyl-1,2-Hexadecanediol (IVa)

To 1,2-hexadecanediol (258 mg, 1 mmol) in chloroform (20 ml) containing perchloric acid (70%, 0.2 ml) was added hexadecanoic anhydride (494 mg, 1 mmol) in small portions over a period of 45 min under stirring. After stirring for 16-17 hr at room temperature, the chloroform was stripped off under a stream of N_2. The residue was treated with 5% aqueous KOH and extracted with ether. Ether extracts were washed free of alkali and dried over anhydrous sodium sulfate. TLC (hexane/ether 70:30) of the reaction product showed the presence of four components characterized as 1,2-O-dihexadecanoyl-1,2-hexadecanediol (42%); a mixture of 1- and 2-hexadecanoyl-hexadecanediols (IIIa and IVa, 45.8%) and 1,2-hexadecanediol (11.8%). These components were separated from each other by column chromatography. The reaction product (620 mg) was applied to a column packed with 30 g silicic acid (100 mesh, Mallinckrodt). 1,2-O-Dihexadecanoyl-1,2-hexadecanediol (258 mg) was eluted with benzene (275 ml); partially acylated 1,2-hexadecanediols (IIIa & IVa 279 mg) with chloroform (600 ml) and the residual 1,2-hexadecanediol (72 mg) was eluted with methanol (100 ml). 1- and 2-O-Hexadecanoyl-1,2-hexadecanediols were finally separated from each other by TLC (hexane/ether 70:30). Position 1-isomer showed a slightly higher Rf (0.49) value than position 2-isomer (Rf 0.38). IR and NMR spectral data for these two isomers given below are in agreement with the proposed structures.

1-O-Hexadecanoyl-1,2-hexadecanediol (IIIa): IR 3447 (OH) and 1709 cm^{-1} (C=O); NMR: δ 0.89 (CH_3, t), 1.25 (CH_2 chain, apparent singlet), 2.2 ($COC\underline{H}_2$, t) 3.60 (CHOH, m), 3.82 (HCHOCOR, dd, Jvic = 7 Hz, Jgem = 11 Hz), 4.0 (HCHOCOR, dd, Jvic = 3.5 Hz, Jgem = 11 Hz). Jones' oxidation of the compound afforded a keto-ester (IX), thus further confirming the proposed structure. IR and NMR spectral data for the keto-ester (IX) are given as follows. IR: 1732 cm^{-1} (C=O); NMR: δ 0.87 (CH_3, t), 1.23 (CH_2 chain, apparent singlet), 2.3 (q, two overlapping triplets, $COCH_2$), 4.47 ($OCCH_2OCO$, S).

2-O-hexadecanoyl-1,2-hexadecanediol (IVa): IR 3447 (OH) and 1739 cm^{-1} (C=O). NMR data are given in Table I.

Preparation of 14-Hexadecanoyloxy-Tetradecanoic Anhydride (V)

1,14-Tetradecanediol (0.53 g, 2.3 mmol) was treated with 1 equivalent of hexadecanoic anhydride (1.23 g, 2.48 mmol) in chloroform (20 ml) containing 1% perchloric acid (70%) as catalyst. Hexadecanoic anhydride was added in small portions during 1 hr under stirring at room temperature and the stirring was continued overnight (18 hr). Chloroform was removed in a stream of N_2. The reaction product was taken up in ether, washed with 5% KOH and with water, and dried over anhydrous sodium sulfate. TLC [hexane/ether 70:30 of the reaction product (1.54 g)] revealed the presence of three components corresponding to residual 1,14-tetradecanediol and its mono- (Rf 0.38) and diacylated (Rf 0.8) products. The monoacylated product was isolated by column chromatography on silicic acid (30 g, 2 x 22 cm). Diacylated (1.1 g) and monoacylated (0.352 g; yield 22%) products were eluted with benzene (230 ml) and chloroform (500 ml), respectively. Residual 1,14-tetradecanediol (0.08 g) was finally eluted with methanol (100 ml).

IR of the monoacylated product exhibited absorption for hydroxyl at 3458 and 3277 cm^{-1} and for ester carbonyl at 1734 cm^{-1}. NMR spectrum displayed signals at: δ 0.87 (CH_3, t), 1.3 (CH_2 chain, apparent singlet), 2.2 ($COCH_2$, t), 3.54 ($C\underline{H}_2OH$, t), 4.00 (CH_2OCOR, t).

14-Hexadecanoyloxy-1-tetradecanol, the monoacylated product, was oxidized by Jones' method (3). The oxidation was carried out at 40 C because of the insolubility of the starting material in acetone at room temperature. After stirring for ½ hr, the reaction mixture was diluted with water and the reaction product was extracted with ether. Ether extracts were washed free of acid and dried over anhydrous sodium sulfate. TLC (hexane/ether 50:50) of the reaction product confirmed the completion of oxidation. IR: 3534 and 3279 (OH), 1731

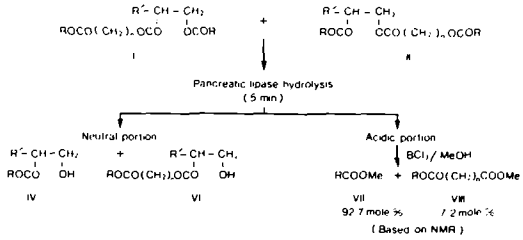

FIG. 2. Pancreatic lipase hydrolysis of triester wax derived from the skin of the rhino mutant mouse.

(OCOR) and 1711 cm^{-1} (COOH).

14-Hexadecanoyloxy-tetradecanoic acid (340 mg, 0.75 mmol) in dry CCl_4 (7.5 ml) was treated with dicyclohexylcarbodiimide (128 mg, 0.62 mmol in 2.5 ml CCl_4) and the reaction mixture was allowed to stand at room temperature for 24 hr (4). The precipitated material (77 mg) was filtered out and the filtrate was evaporated in a stream of nitrogen to recover 14-hexadecanoyloxy-tetradecanoic anhydride (V). The anhydride was purified by repeated crystallizations from acetone (191 mg). IR: 1813 and 1731 cm^{-1}. In contrast to fatty acid anhydrides, absorption at lower frequency was relatively stronger than that at higher frequency because of its overlapping with ester carbonyl absorption.

Lipase Hydrolysis of Triesters

Since IR and NMR spectra of both isomeric model compounds Ia and IIa were found identical to that of naturally occurring triesters, a distinction between two possible structures I and II could not be achieved by comparison of IR and NMR spectra. An attempt was therefore made to partially hydrolyze the sample using pancreatic lipase (Fig. 2). In a typical experiment, 10.5 mg of triesters was treated with 11.2 mg of crude pancreatic lipase (Sigma, St. Louis, MO), 1 ml of tris buffer (pH 8), 2.5 ml $CaCl_2$ (2.2%) and 1 ml of deoxycholic acid (0.05%) and the reaction mixture was shaken for 5 min (8). The reaction mixture was immediately transferred to ice bath and extracted three times with 5 ml of diethyl ether. The combined ether extracts were washed with water and dried over anhydrous sodium sulfate. The hydrolyzate (9.8 mg) was freed from unhydrolyzed triesters by TLC (hexane/ether 95:5) and the partially hydrolyzed sample (3.4 mg) was divided into acidic (1.3 mg) and neutral (2.0 mg) portions on alkaline silicic acid column (9). This procedure was repeated several times. The neutral portion (16.6 mg) was resolved into two components after silylation. These were separated by TLC (hexane/ether 95:5, Rf 0.4 and 0.34, respectively). Individual fractions were hydrolyzed to regenerate hydroxy compounds. These two components from neutral portion were characterized as 2-O-acyl-1,2-alkanediols (IV, 5.4 mg) and 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols (VI, 11.0 mg), respectively. IR (IV): 3574 (OH) and 1734 cm^{-1} (C=O); IR (VI): 3496, 3471 (OH) and 1738, 1691 (C=O). NMR spectral data for IV and VI are given in Table I. The acidic part (22 mg) obtained by lipase hydrolysis was methylated with BCl_3/MeOH (10) and was separated into two components by TLC (hexane/ether 95:5). These two components were characterized as fatty methyl esters (VII, 14.5 mg) and ω-acyloxy fatty methyl esters (VIII, 7.1 mg). NMR spectral data for VII and VIII are shown in Table I.

RESULTS AND DISCUSSION

Neutral Portion—Identification of 2-O-Acyl- and 2-[(ω-Acyloxy)O-Acyl] 1,2-Alkanediols (IV and VI)

TLC of the neutral portion (hexane/ether 60:40) showed a diffused spot moving essentially in the region of monohydric fatty alcohols. No 1,2-alkanediols were observed indicating that no portion of triester wax had undergone complete hydrolysis. Chromic acid/pyridine (2) oxidation of the sample did not yield any ketoester (based on NMR) showing that only primary ester linkage was cleaved by lipase hydrolysis. This observation agrees with the behavior of pancreatic lipase on triacylglycerols.

NMR spectrum (Table I) of the relatively faster moving component (IV) showed methylene protons of glycol moiety at δ 3.43 and 3.52 (dd). This represents an upfield shift of these protons relative to triester wax in which the corresponding protons resonate at δ 3.85 and 4.15 (dd). Thus, it was apparent that primary ester linkage cleaved by pancreatic lipase was a part of 1,2-diol moiety. Alkaline hydrolysis of this fraction afforded fatty acids and 1,2-alkanediols. This fraction of the neutral portion was thus comprised of 2-O-acyl-1,2-alkanediols.

NMR spectrum (Table I) of the relatively slower moving fraction (VI) of the neutral portion was essentially similar to that of faster moving fraction except that an additional triplet was observed at 3.94 ppm. Alkaline hydrolysis of the sample yielded ω-hydroxy fatty acids, fatty acids and 1,2-alkanediols. This fraction of the neutral portion was thus comprised of 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols.

Acidic Portion—Identification of Fatty Acids and ω-Acyloxy-Fatty Acids

Acidic portion, after methylation (10), was resolved into two components by TLC. Relatively faster moving component showed NMR spectrum (Table I, VII) and Rf value identical to those of fatty acid methyl esters. The relatively slower moving component yielded fatty acids and ω-hydroxy fatty acids on alkaline hydrolysis suggesting that this component was composed of ω-acyloxy fatty acids. NMR spectrum (Table I, VIII) was in complete agreement with ω-acyloxy methyl esters. A triplet at 3.94 ppm is assigned to methylene protons adjacent to acyloxy group. Final confirmation of the structure was achieved by comparing NMR spectrum of the relatively slower moving component with that of methyl-14-hexadecanoyloxy-tetradecanoate (X). The latter was prepared by partial acylation of 1,14-tetradecanediol with hexadecanoic anhydride followed by Jones' oxidation and methylation.

It has been shown that triester wax on lipase hydrolysis affords 2-O-acyl-1,2-alkanediols, 2-[(ω-acyloxy)O-acyl]-1,2-alkanediols, fatty acids and ω-acyloxy fatty acids. Formation of all these products, assuming that no acyl migration occurs, indicates an isomeric mixture of both possible structures I and II for triesters. Using our model compounds III and IV, it was established that no acyl migration occurs and the reaction is specific for primary ester grouping of 1,2-diol moiety under the experimental conditions used. This specificity of pancreatic lipase was also observed with 1,2-alkane diol diesters. However, when a longer reaction time of ½ hr-12 hr was given, acyl migration to varying degrees was observed. Assuming that enzyme has no particular preference for fatty acid chain lengths, the proportions of isomers I and II can be computed from the molar ratios of fatty acids and ω-acyloxy fatty acids. The molar percentage of ω-acyloxy fatty acids in a mixture with fatty acids, originating from isomer II and I, respectively, in pancreatic lipase hydrolysis, was determined by NMR as 7.2 mole %. ω-acyloxy methyl esters display a triplet at 3.94 δ for methylene protons adjacent to acyloxy group (Table I, VIII). Let x represent the area at 3.94 δ associated with OCH_2 protons, and y represent the total area associated with α-methylene protons of fatty acids and acyloxy fatty acids combined. Since acyloxy fatty acids contain two pairs of α-methylene protons, while fatty acids contain only one pair, the contributions to y of the two types of compounds are equal to 2x and to y-2x, respectively. Thus, the molar concentrations of the two classes are represented by x and by y-2x, respectively, and the relative (percent) concentration of acyloxy acids equals $100x/(y-2x+x) = 100x/(y-x)$. Weights of lipase hydrolysis products were not used to calculate proportions of isomers because of ambiguity involved in estimating average molecular weight of a mixture having wide range of carbon chain lengths (1).

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The Effect of a Short Term Saturated Fat Diet on the Apoprotein Composition and Radioiodination Properties of Rat Very Low Density Lipoproteins

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ABSTRACT

The effect of a saturated fat diet on the apoprotein composition and radioiodination properties of plasma very low density lipoprotein (VLDL) was studied in rats. After feeding the diet for 10 days, the proportion of ^{125}I attached to VLDL lipid decreased from 50% (control animals) to 8%, the remainder (92%) being bound to the apoportein components. The decreased lipid labelling was associated with proportional changes in the fatty acid composition of serum and VLDL lipids, the most notable change being a reduction in linoleic acid (30-8%) content which occurred in all the major lipid classes of both serum and VLDL. Analysis of VLDL after radioiodination showed that most of the radioactivity incorporated into the lipid moiety was associated with phospholipid. The proportion of ^{125}I bound to phospholipid decreased after feeding rats a saturated diet. The proportion of soluble (small molecular weight peptides and arginine rich peptide) to insoluble (B apoprotein) did not alter during the saturated fatty acid dietary regime and no differences in the distribution of soluble proteins were observed. It is concluded that feeding a saturated fat diet to rats for 10 days significantly improved ^{125}I labelling of the apoprotein moiety while apparently not inducing changes in apoprotein composition.

INTRODUCTION

As the apolipoprotein components of plasma very low density lipoproteins (VLDL) become better characterized, more information is obtained about their structure, function (1-4), and distribution. It has been suggested that in certain cases of hyperlipoproteinaemias or other abnormalities (5) and after feeding diets with a high sucrose content (6), alterations in the proportion of apolipoprotein subunits may occur.

In a previous communication (7), we showed that increased labelling of VLDL protein could be achieved only by manipulation of the iodine monochloride technique of McFarlane to an extent which consequently raised the iodine: protein ratio to a level unacceptable for in vivo turnover studies. Consequently, it was necessary to alter the proportion of saturated:unsaturated fatty acids in rat VLDL in order to reduce the proportion of label in (bound to) VLDL lipid following radioiodination and to obtain preparations with a low iodine-protein ratio suitable for in vivo investigation. However, since other dietary manipulations have been alleged to alter the subunit composition of VLDL apolipoprotein (6), we investigated the effect of short term saturated fat feeding on rat VLDL apolipoprotein composition and radioiodination properties.

MATERIALS AND METHODS

Two groups of male wistar rats of the John Curtin School strain weighing 300 g were used in these studies. One group was fed continuously on a commercial (control) diet of rat pellets (Mecon Agricultural Products, Eastwood, Melbourne, Australia), and the second group was fed for various times on a saturated fat diet consisting of 18% protein as casein, 72.5% carbohydrate as sucrose, 5% fat (dripping or lard) 0.23% D-L methionine and a standard vitamin (0.27%) and mineral (4%) supplement similar to that of the control diet. All rats were allowed food and water ad libitum. At the end of the feeding time the, rats were fasted for 6-8 hr or 16 hr anaesthetized with ether, and bled from the abdominal aorta at the same time each day (9-11 a.m.) to avoid differences due to diurnal variation.

Isolation and Iodination of VLDL

After centrifuging the clotted blood at 2,500 rpm for 30 min at 4 C in a MSE Mistral 6L, ethylenediametetra-acetic acid, disodium salt, (EDTA) was added to each pooled serum sample to give a final concentration of 1 mM. Five ml of serum was added to Spinco 40.3 tubes and overlaid with 2.0 ml 0.15 M NaCl (pH 7.4) containing 1 mM EDTA at density 1.006 g/ml. After centrifugation at 12 C for 16 hr at 39,000

rpm, the VLDL was isolated by slicing the tubes and purified by two further centrifugations, one for 8 hr at 12 C in a spinco 50 rotor at 50,000 rpm and finally for 16 hr at 12 C in the 40.3 rotor at 39,000 rpm.

Iodination of VLDL with ^{125}I (Na ^{125}I Radiochemical Centre, Amersham) was performed at pH 10.0 using the method of McFarlane as described previously (7). Protein estimations were performed using the Lowry Method (8).

Delipidation and Polyacrylamide Gel Electrophoresis

VLDL to be delipidated was dialyzed against 5 mM NH_4HCO_3 (pH 8.2) for 24 hr and lyophilised. The lyophilised lipoprotein was then suspended in 200 µl of 5 mM NH_4HCO_3 pH 8.2 and delipidated by adding 2 ml methanol, and after mixing, 2 ml of chloroform using the same procedure described previously (9). After drying under nitrogen, the protein was dissolved in 0.1 M SDS in 0.1 M Triz-HCl buffer pH 8.2. Prior to loading on polyacrylamide gels, the concentration of SDS was reduced to 0.05 M by the addition of 0.01 M Triz-HCl, pH 8.2 and solid urea added to a final concentration of 8 M.

The stained gels were then scanned under identical conditions using a Varian Techtron gel scanner and the areas under the absorption peaks determined (11). The densitometric relationship was linear over the range of peptide concentrations measured and was similar to that described by Kane et al. (12).

Separation of Soluble from Insoluble Proteins

Since the protein moiety of rat VLDL contains a high molecular weight protein (insoluble and homologous to the human B protein) and a soluble protein complex, which includes the small molecular peptides homologous with the C peptides of human VLDL (13), the following procedure was used for their separation.

After delipidation in 5 ml delipidating tubes, 0.5 ml of 5 mM NH_4HCO_3 was added to the dried protein; the tubes were then vortexed and allowed to stand overnight. The tubes were then centrifuged at 2,000 g for 30 min and the supernatant taken off and retained. The pellet was then washed with a further 0.5 ml of 5 mM ammonium bicarbonate, vortexed, and recentrifuged. The supernatant of this spin was combined with that of the first spin and represents the soluble portion. The insoluble protein (pellet) was solubilized in 0.1 M NaOH for protein estimation or 0.1 M SDS in 0.1 M Triz-HCl pH 8.2 for PAGE. The soluble portion was diluted to a known volume with 5 mM NH_4HCO_3 pH 8.2 and the protein concentration determined (8). Validation of this method will be described elsewhere.

Lipid Analyses

Triglyceride and cholesterol were estimated using aTechnicon Autoanalyser II [Autoanalyser Methods 24a and N78 (1965) for triglyceride and cholesterol respectively]. The lipids of an aliquot of radioiodinated VLDL (plus 30 mg of concentrated human serum albumin as protein carrier) were extracted using 6 ml chloroform-methanol (2:1) and phase separated by the addition of one-fifth the volume of 0.9% saline (14). The lipids obtained by evaporation of the chloroform phase under N_2 were separated by thin layer chromatography on silica gel using hexane, diethyl ether, methanol, and acetic acids in the proportions 180:40:6:4, respectively, as solvent. Cholesteryl ester, triglyceride, free fatty acid, and cholesterol bands were scraped off the plates and eluted with 20 ml diethyl ether. Phospholipid was eluted as described by Skipski et al. (15), eluting twice with chloroform-methanol-acetic acid-water (25:15:4:1.9) then washed with methanol and methanol-acetic acid-water (94:1:5). Lipid fractions were then evaporated under N_2 and dissolved in ethanol. An aliquot from each sample was radioassayed for ^{125}I using a Packard Autogamma Spectrometer. Where necessary, corrections for quenching were made by reference to prepared quench curves.

Lipids from samples for gas liquid chromatographic analyses were extracted using chloroform-methanol. After phase separation (14) and evaporation of the chloroform layer under N_2, the methyl esters were prepared by adding 4 ml 4% sulphuric acid in dry methanol and standing overnight. After the addition of an equal volume of water and heptane (5 ml), the samples were shaken and the heptane layer removed and evaporated down to 100 µl under nitrogen.

Gas liquid chromatographic analyses were performed on a Packard Gas Chromatograph (Model 824) with nitrogen as carrier gas. Separations were carried out on a circular 1.5 m x 4 mm ID column packed with 13% ethyleneglycol adipate on 80-100 mesh Gas Chrom Chromosorb P (Applied Science Laboratories, Inc., State College, PA). The column was operated at 190 C and at 40 p.s.i. inlet pressure. For identification of fatty acids, retention times relative to methyl stearate were compared to those of pure reference compounds and to National Institutes of Health (USA) (NIH) type mixtures (16) D-99 (14:0, 16:0, 16:1, 18:0, 18:1) and D-104 used as a quantitative aid

TABLE I

Effect of the Saturated Fat Diet on Very Low Density Lipoprotein (VLDL) Lipid Fatty Acid Composition[a]

Fatty acid	Control VLDL			Saturated VLDL (10 days)		
	PL	TG	CE	PL	TG	CE
	% Composition[b]					
16:0	23.2	26.6	25.9	22.2	23.2	13.8
16:1	1.4	2.9	2.0	3.3	8.0	12.5
18:0	25.3	6.3	15.6	21.0	7.3	9.1
18:1	11.9	29.9	30.2	22.5	52.4	51.5
18:2	24.8	29.0	17.3	11.2	7.8	6.2
18:3	tr	2.6	2.8	3.4	1.5	tr
20:1[c]	tr	1.1	1.2	tr	tr	0.5
20:4	13.2	1.5	5.0	15	tr	6.1
20:5	tr	n.d.	n.d.	1.2	n.d.	n.d.

[a]PL = Phospholipid, TG = Triglyceride, CE = Cholesteryl ester, n.d. = not detected. Significant amounts of 14:0 were not detected. Trace amounts of some unidentified fatty acids were also observed.

[b]Figures represent the mean of two determinations on pooled sera from 20 male rats (fasted for 6-8 hr).

[c]Tentative identification.

(14:0, 16:0, 18:0, 18:1, 18:2, 18:3, 20:0, 22:0, 22:1, 24:0), (Serdary Research Laboratories, London, Ont. Canada).

The distribution of the fatty acids was determined after calculating the peak areas with an Infotronics Digital Integrator (Model CRS-208) and also by hand, by measuring the retention time (distance from solvent front, mm) and height of the peak (mm). The relative differences between replicate analyses were less than 2% for the major components and 0.1-3% for the minor components (2-10% of the total mixture). Results using the integrator agreed with those calculated by hand (<2% difference).

RESULTS

A study by McLeod (17) has already shown that rats fed on the saturated fat diet exhibit similar growth curves to rats fed on the commercial rat chow. Fatty acid analyses of different lots of beef dripping and of the saturated fat diet in which beef dripping was the only fat added showed that there was little variation in the fatty acid composition. A comparison of the fatty acid composition of the control and saturated fat diet revealed that the control diet has a much higher content of linoleic acid (18:2), 31.8 ± 1.9% compared to 3.1 to 8.3% in the saturated fat diet and oleic acid (18:1), 32.0 ± 1.1% in control and 45.5 to 49.9% in the saturated fat diet. The degree of saturation of the saturated fat diet is therefore considerably higher than that of the control diet.

Effect of the Saturated Fat Diet on Serum and VLDL Fatty Acid Composition

No significant difference in triglyceride and cholesterol concentration was observed in rats fed the saturated fat diet and control animals. Changes in total serum and VLDL fatty acids after 10 days on the saturated fat diet reflected that of the diet. Notable changes occurred in the linoleic acid (18:2) content, which fell from 29.9 to 9.7% in serum and 29.6 to 7.5% in VLDL. This decrease was accompanied by increases in both oleic (18:1) and palmitoleic acids (16:1). This trend was seen in each of the major lipid classes (phospholipid, free fatty acids, triglycerides, and choelsteryl esters) in serum (not shown) and VLDL (Table I). No other major changes were observed. The content of arachidonic acid was highest in cholesteryl esters of serum and phospholipids of VLDL.

The rate at which the change in serum fatty acid composition occurs after commencement of the saturated fat diet was determined. Rats were fasted for 16 hr before exsanguination. Under these conditions, a gradual decrease in 18:2 from 26% on the control diet to 15.6% after 10 days on the saturated fat diet and a slight increase in the content of 16:1 (2% to 7%) was observed. The decrease in 18:2 was significant after only 24 hr on the saturated fat diet ($p<.01$). A slight decrease in 18:3 content and increase in 18:1 was also observed in the 10 days. There was no change in arachidonic acid.

Rats fed the saturated fat diet for 1 to 10 days but fasted for only 4-5 hr prior to exsanguination exhibited major changes in both serum and VLDL after 24 hr on the diet. Linoleic acid content dropped from 25% in control to 12% in 24 hr. This was reflected by increases in palmitic (16:0), palmitoelic (16:1), and oleic (18:1) acids.

Effect of the Saturated Fat Diet on the Distribution of Radioactive Iodine in Radioiodinated VLDL

The proportion of ^{125}I binding to lipid progressively decreased after rats had been fed the saturated fat diet (Table II). VLDL from saturated animals (10 days) was consistently labelled at efficiencies of 8-12% with 94 ± 2.7% (S.D.) of the label in the protein moiety, 5.2 ± 2.9% associated with lipid and 0.8 ± 0.76% in the free state. The I/P ratio varied from 0.5-1.5 in these preparations.

Analysis of radioiodinated VLDL lipid by thin layer chromatography showed that between 84 and 96.5% of the control and 78.7 and 87.1% of the saturated VLDL lipid ^{125}I was found in the phospholipid fraction. The

percentage distribution of ^{125}I bound to lipid, protein and free in these samples ranged from 59.2-56.3: 39-40.1: 1.8-3.4 in control and 3.5-6.2: 90.6-96: 0.5-3.2 in saturated VLDL.

Effect of the Saturated Fat Diet on the Apoproteins of VLDL

In order to detect possible changes in the VLDL apoprotein composition, rats were killed at varying times from 1-9 days after commencement of the saturated fat diet. Their plasma VLDL apoproteins were then isolated and separated by polyacrylamide gel electrophoresis (PAGE) and the stained bands were divided into four zones as described by Fidge and Poulis (9). No apparent differences in the VLDL protein composition were observed or measured after scanning the gels, and computing the ratio of the area of Zone I (Insoluble protein):Zone II + III + IV (Soluble proteins). Further analysis of the soluble proteins by densitometry revealed no change in the proportion of apolipoprotein C to other soluble apolipoproteins (11).

The proportion of the soluble to insoluble apolipoproteins of VLDL from rats fed the control or saturated fat diet for 10 days is shown in Table III. Values for saturated VLDL did not vary significantly from the controls. Thus, the short term feeding of a saturated fat diet did not apparently affect the proportion of B to C apoproteins in rat VLDL.

DISCUSSION

The radioiodination properties of VLDL obtained from animals fed a saturated fat diet for 10 days were changed such that over 90% of the ^{125}I was consistently bound to the protein of VLDL when the I/P ratio was <1. Therefore, the VLDL was essentially radioactively labelled only in the protein. Since the VLDL was to be used in metabolic studies, it was essential to determine whether the saturated fat diet had changed the apoprotein composition, in view of the findings elsewhere (6) that administration of a high sucrose containing diet resulted in pronounced increases in the proportion of C apolipoproteins. In the studies reported here, the saturated fat diet contained 72% sucrose which apparently substituted for starch in the commercial chow. However, since the serum concentrations of triglyceride and cholesterol

TABLE II

Effect of a Saturated Fat Diet on the Iodination of Very Low Density Lipoprotein (VLDL)[a]

No. of days on diet	% Distribution of label[b]		
	Lipid	Protein	Free
Control (0)	44.5 ± 6	52.2 ± 5	3.3 ± 1.0
2	40.6	56.7	2.7
4	21.7	72.7	5.6
6	21.6	74.0	4.4
8	12.4	84.2	3.4
10	8.2	91.4	0.8

[a]VLDL containing 0.5 mg protein was used in each iodination.

[b]Figures represent the mean of two determinations except for the control which represents the mean of three determinations ± S.D.

TABLE III

Comparison of the Proportion of Ammonium Bicarbonate (5 mM) Soluble and Insoluble Apoprotein Present in Rat Very Low Density Lipoprotein (VLDL) from Control and Saturated Diet Fed Rats, Following Delipidation with Organic Solvents

Sample		Percent insoluble	Percent soluble	Number of samples	% Recovery of protein after delipidation[a]
Control VLDL	(a)	38.7 ± 11.9[b]	61.5 ± 11.7	8	82.5 ± 9.5
	(b)	32 ± 12.0	68 ± 11.8	16	86 ± 8.4
Saturated VLDL (10 days)		36.1 ± 5.2	63.9 ± 5.3	6	85 ± 6.3

[a]and after 5 mM ammonium bicarbonate separation.

[b]Values represent the mean ± S.D. Figures for the saturated VLDL (from rats fasted for 8-10 hr) did not differ significantly from the control VLDL (from rats fasted for 4-16 hr) as determined by students 't' test.

		Insoluble	Soluble
Probability	(%)		
	(a)	66	67
	(b)	46	45
't'	(a)	.46	.44
	(b)	.77	.79

Differences are significant when P<5% (i.e. .05) and t > 2.10.

reamined steady throughout the 10 day period, and since the apolipoprotein composition remained unaltered, there was little evidence of carbohydrate-induced hyperlipoproteinaemia during the short term saturated fat diet. We are unable to compare the different observations between the present experiments and those reported by Roheim et al. (6) since the composition and duration of the high sucrose diet were not published in that report.

In earlier metabolic studies (9,11), it has been shown that there was no significant difference in the disappearance from the serum of ^{125}I labelled VLDL obtained from rats fed the saturated fat diet for 2, 4, and 8 days when injected into control diet fed rats. Kinetics of disappearance of ^{125}I from VLDL apolipoprotein were similar to that of the whole lipoprotein (9,11).

The fatty acid composition of the serum of fed rats is thought to remain stable throughout the day, other than a peak at 3 and 6 pm (18). Even so, rats used in these experiments were bled at the same time each day. Changes in the fatty acid composition of the serum and VLDL reflected the changes in the diet, the major change occurring in the essential fatty acid linoleic acid (18:2) of all lipid classes. The proportion of major fatty acids found in control serum phospholipids, triglycerides and cholesteryl esters compare well with those obtained by Aftergood and Alfin-Slater (19) and Sgoutas (20) for untreated rats.

Analysis of the radioiodinated VLDL lipid revealed that most of the ^{125}I was incorporated into the phospholipid of VLDL from control and saturated fat fed rats. This is not surprising since phospholipid is thought to be a surface constituent rather than in the core like nonpolar lipids (21).

The fact that most of the radioactivity associated with the VLDL lipid moiety was bound the phospholipid suggested that any reduction in the unsaturated fatty acids of this lipid class might result in decreased lipid labelling of the whole molecule. Thus, the evidence for the reduction in linoleic acid content of phospholipid together with the fivefold reduction of ^{125}I associated with the VLDL lipid moiety after saturated fat feeding for 10 days, suggests that saturation of VLDL phospholipid acyl groups was the major reason for improved protein radioiodination. We have concluded that this method is a satisfactory means of providing rat VLDL labelled essentially in the protein moiety alone without any apparent alteration of the apoprotein subunit composition.

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BF_3-MeOH: A Single Reagent for Ozonolysis of Monoethylenic Unsaturation

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ABSTRACT

Ozonolysis of monoethylenic fatty acids in BF_3-MeOH, or in MeOH with subsequent addition of BF_3-MeOH, gives oxidative fission acid products as methyl esters in a nominal 100% yield and a purity ≥98% of principal acid products. The in situ esterification step requires ca. 1 hr of heating with 7 or 14% BF_3, but other time requirements are much less, totalling no more than half an hour. Different liquid phases for open-tubular gas liquid chromatography of some products are compared.

INTRODUCTION

Ozonolysis has always been an extremely attractive mode of oxidative fission because of the practically instantaneous and total reaction of ozone with ethylenic unsaturation (1-3), and the avoidance of inorganic and nonvolatile oxidants. The practical problem of incomplete decomposition of ozonides has been largely overcome in the direction of acidic products by using participating solvents such as methanol, and peracids such as performic acid formed in situ (4,5). In the direction of neutral products, commonly aldehydes, the most popular trend is the use of reducing agents such as triphenylphosphine (6,7) or dimethyl sulfoxide (8-10), or catalytic hydrogenation (11,12). Numerous other modifications of ozonolysis technology are in the literature (1). One remaining problem in the formation of acidic products has been the need to recover the acidic products and convert them to methyl esters, although this can be done in a homogeneous solution with 2,2-dimethoxypropane (13). Although oxides of fluorine have powerful oxidizing properties (14), lipid chemists are normally more familiar with fluorine in the widely-used BF_3-methanol esterification reagent (15,16).

In the range of 1-10 mg sample, the oxidative scission of common C_{18} monoethylenic acids under convenient ambient conditions, proceeds smoothly, with total conversion to acidic products, as methyl esters, by heating with BF_3-methanol. The exact roles of methanol, ozone, and BF_3 in the oxidizing step are still under investigation in this laboratory. However, the esterification step is probably conventional for the BF_3-MeOH reagent since times for production of diacid ester product seem to be normal.

EXPERIMENTAL PROCEDURES

Ozonolysis

The simplest procedure, using 1-10 mg of sample, is carried out as follows: weigh appropriate amounts of elaidic acid (Hormel Institute, Austin, MN) and an internal standard saturated acid, into a 10 ml screw cap centrifuge tube such as a Corning No. 8142, with a mated leak-tight cap with a Teflon-faced rubber liner. Add 2 ml of 14% BF_3-MeOH reagent (Applied Science Laboratories, State College, PA, No. 18017, ampoules), and bubble in ozone briskly for 1 min (Welsbach Model T-408, oxygen flow 300 ml/min at 95V). Cap and transfer to a heater block (nominal 100 C) for 1 hr. Cool, add 2 ml H_2O and 2 ml of methylcyclohexane, and agitate briskly. Transfer 1.9 ml of hydrocarbon layer to a 5 ml glass-stoppered centrifuge tube and reextract H_2O/MeOH layer with 2 ml of hydrocarbon. Add 1.9 ml of the second extract to the first extract and wash with 1 ml of distilled water. Transfer most of the hydrocarbon layer to a second glass-stoppered centrifuge tube and, if required, concentrate with a stream of nitrogen and a near-boiling water bath.

For experiment I (Table I), elaidic and suberic acids were weighed and dissolved in reagent grade MeOH. First, a 1 ml aliquot of this solution (containing ca. 4-5 mg of each acid) was esterified with 1 ml of 14% BF_3-MeOH for 30 min (no ozone exposure), processed for recovery of the esters as described above, and analyzed by gas liquid chromatography (GLC) (results I-A). Then, a second aliquot was exposed to ozone for 1 min, 1 ml of 14% BF_3-MeOH was added, the solution was heated for 60 min, and the esters were recovered as described above (results I-B). Finally, 1 ml of 14% BF_3-MeOH was added to a third aliquot before ozone treatment, as suggested above, and the esterification and ester recovery were the same as for I-B (results I-C).

For experiment II, a sample of ca. 1 mg of methyl ester of synthetic *cis*-docos-11-enoic acid (courtesy of A. Spark, South African

TABLE I

Methyl Ester Yields from BF_3-MeOH Reaction and Gas Liquid Chromatographic-Flame Ionization Detection (GLC-FID) Quantitation of Relative Molar Responses

Experiment	GLC column	Acid used as Reference material	Effective carbon response	Acid examined (primary product only from O_3 studies)	Effective carbon response found	
					Range	Average
I-A	BDS	Elaidic (weighed)	18	Suberic (weighed)	7.0–7.9	7.5
I-B	SILAR-5CP	Suberic (weighed)	7.5	Azelaic (O_3 product)	8.3	
	BDS	Suberic (weighed)	7.5	Azelaic (O_3 product)	8.2–9.2	8.5
I-C	BDS	Suberic (weighed)	7.5	Azelaic (O_3 product)	8.3–8.9	8.6
II	SILAR-7CP	Undecanoic (O_3 product)	11	Undecanedioic (O_3 product)	10.6–10.9	10.8
III	SILAR-5CP	Stearic (weighed)	18	Azelaic (O_3 product)	8.7–8.8	8.7
IV	SILAR-5CP	Stearic (weighed)	18	Azelaic (weighed)	9.2–9.4	9.3[a]
	BDS	Stearic (weighed)	18	Azelaic (weighed)	8.5–9.0	8.8

[a]This group of results based on data including an attenuation change.

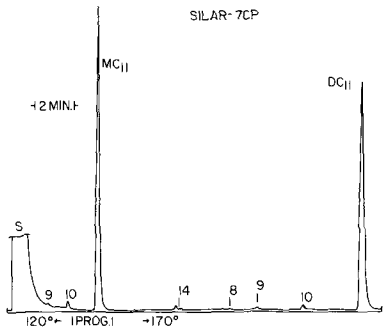

FIG. 1. Methyl ester products of ozonolysis of synthetic *cis*-docos-11-enoic acid (as methyl ester) in BF_3-MeOH. From left to right, 9, 10, 11, and 14 are methyl esters of monocarboxylic acids; 8, 9, 10, and 11 of dicarboxylic acids. Analysis on open-tubular column with SILAR-7CP as the liquid phase. Conditions: 4 min at 120C, program at 24 C/min to 170 C, hold; attenuation 32X.

Fishing Research Institute, Capetown) was ozonized in reagent grade MeOH. A 0.8 ml portion of 14% BF_3-MeOH was added, followed by heating for 1 hr. In this experiment, one objective was to compare the relative mole responses in the flame ionization detector (FID) of the primary mono- and dicarboxylic acid products (Table I). The other objective was to examine the actual purity of the material (Figure 1).

Experiment III was carried out to check the dicarboxylic acid yield from ozonlysis of elaidic acid, using stearic acid as the internal standard. Experiment IV was conducted to check the FID response of dimethyl azelate relative to methyl stearate. Weighed amounts of a mixture of dimethyl azelate and methyl stearate in methylcyclohexane solution were gas chromatographed without further treatment.

Gas Liquid Chromatography

All analyses were carried out with stainless-steel open-tubular columns 150 ft (46 m) in length and 0.01 in (0.25 mm) ID in a Perkin-Elmer Model 900 or 990 gas chromatograph with FID. Liquid phases (Table II) were butanediol succinate polyester (BDS), or SILAR-5CP or SILAR-7CP (Applied Science Laboratories). Peak areas (1 mv recorder) were measured by either Disc Instruments ball and disc type integrators or Linear Instruments Corp. stepping integrators. Quantitative comparative studies of C_8-C_{18} monocarboxylic and various dicarboxylic acid products in one GLC analysis were based on temperature programming with the following scheme being satisfactory:- 100 C for 4 min, program at 24 C/min to 170 C or 180 C and hold.

TABLE II

Equivalent Chain Length (ECL) Values of Dicarboxylic Acid Dimethyl Esters and Iso Esters on Open-Tubular Columns Coated with Different Liquid Phases

Dicarboxylic acid	Liquid phase and temperature		
	BDS (170 C)	SILAR-5CP (180 C)	SILAR-7CP (170 C)
C_8 Octanedioic (Suberic)	–	–	15.98
C_9 Nonanedioic (Azelaic)	15.79	15.82	16.98
C_{10} Decanedioic (Sebacic)	16.80	16.83	17.95
C_{11} Undecanedioic	17.80	17.83	18.96
C_{12} Dodecanedioic	18.77	18.84	19.95
C_{13} Tridecanedioic (Brassylic)	19.79	19.84	20.97
C_{14} Tetradecanedioic	20.78	20.84	--
C_{15} Pentadecanedioic	21.75	21.86	–
Iso 20:0(18-methylnonadecanoic)	–	19.49	19.48
Iso 22:0(20-methylheneicosanoic)	21.57	–	–

The basic problem in evaluating the results of oxidative fission, the quantitative behavior of the flame ionization detector (17-20), was investigated with the apparatus and columns used for ozonolysis products. Units for relative molar responses are given as "effective carbons" (i.e., a methyl or methylene carbon is taken as 1) (17).

RESULTS AND DISCUSSION

Calibration studies (Table I) showed that the effective carbon responses varied slightly with the liquid phase, or less probably with the fact that different open-tubular columns were used in different GLC units. As shown in Table I, the nominal dimethyl ester response for the lower C_n acids was $C_{n-0.5}$ carbons. This value was used in calculating ozonolysis results relative to internal standards.

Relative to stearic acid methyl ester in the final product solution, the principal C_9 dimethyl dicarboxylic acid product (dimethyl azelate) from the ozonolysis of elaidic acid amounted to at least 95-100% yield of theory when the effective carbon response was taken on a relative molar basis as 18 for methyl stearate and 8.5-8.8 for dimethyl azelate. The most obvious dicarboxylic secondary oxidation product (C_8) was 1-2% of the principal C_9 product, and the C_7 and C_6 dicarboxylic acid byproducts were an order of magnitude less. Comparable byproducts of C_{11} principal products from ozonolysis of *cis*-docos-11-enoic acid are illustrated in Figure 1. The monocarboxylic acid products were similar insofar as the apparent yield and secondary products were concerned. Provided the hydrocarbon solvent was free of moderately volatile impurities, the procedure outlined produced no products detectable by GLC in blank runs of the whole procedure. SILAR-5CP was preferred for programming since at 16X attenuation the baseline rise for SILAR-5CP (or SILAR-7CP) columns was $\leqslant$1% of the 1 mv recorder scale, but with BDS the rise amounted to as much as 40% of the 1 mv chart span. The virtual absence of other volatile products under these gas liquid chromatographic conditions (Figure 1) further suggests that this oxidative fission system gives a 100% yield of mono- and dicarboxylic acids. The virtual absence of secondary products from ozonolysis of monoethylenic acids facilitated detection of small impurities such as a docos-8-enoic acid impurity in the synthetic 11-docosenoic acid found at the 0.1% level, without concentration, by recognizing the C_{14} monocarboxylic acid product and excessive C_8 dicarboxylic acid product (Figure 1). Subsequently, this trace acid impurity was isolated by TLC-($AgNO_3$) and identified as the *trans* isomer.

Attempts to make the procedure more flexible by carrying out the ozonolysis in pure reagent-grade methanol (1 ml) at ambient temperature, and then adding an equal volume of 14% BF_3-MeOH to give a ca. 7% solution of BF_3, were apparently equally as successful in product yield and composition as the procedure outlined above, but have not been investigated in depth. The empty volume of the centrifuge tube ($\geqslant$5ml) is normally left filled with O_2/O_3 gas, a mixture which may provide extra oxidant over and above that in the ozonide or in solution, or the MeOH solvent may simply dissolve enough O_3 to provide the necessary oxidant. The necks of the centrifuge tubes eventually became cloudy and fragile, presumably due to attack by HF during the esterification step.

An esterification period of 30 min was often inadequate to give the maximum yield of dicarboxylic acid product with 7% or 14% BF_3 when starting with elaidic acid (i.e., the dicarboxylic acid product required two esterifi-

cation steps), but 60 min appeared to be fully adequate. The choice of methylcyclohexane as solvent was governed by its relatively high boiling point (101 C) (it was, nevertheless, conveniently removed with a nitrogen stream in a boiling water bath), and by the ring structure which apparently made it a better solvent for a range of diesters than linear aliphatic hydrocarbons such as *n*-hexane. An adequate phase separation took only 1-2 min in the first and second extractions from the $MeOH/H_2O$ phase. The water wash of the hydrocarbon phase, which required 5-10 min for both phases to clear, was only a precaution, to prevent carryover of BF_3 hydrolysis products. It also possibly reduced solvent tailing from MeOH in the gas liquid chromatographic analyses. Direct injection of BF_3-MeOH solution was not evaluated with the open-tubular columns, but might be feasible with packed columns.

The GLC analysis of dicarboxylic methyl esters was fastest and most convenient on SILAR-7CP, but as soon as samples containing traces of certain natural fatty acids such as palmitic and stearic were analyzed, problems arose from the coincidence of these with dicarboxylic acid methyl ester products (Table I). The constant fractional chain length (FCL) values for the esters of dicarboxylic acids (Table II) facilitate isothermal identifications for middle-range dicarboxylic acid esters with either BDS or SILAR-5CP and are also useful in temperature programming. The latter liquid phase appears to be ideal for open-tubular analyses of esters of mono- and dicarboxylic acid products, and it also has great thermal stability; however, retention values have not been determined for packed columns. The use of iso acids such as the uncommon iso-20:0 or iso-22:0 acids as internal quantitation standards (Table II) would permit simultaneous determination of saturated acids such as palmitic, stearic, etc. along with the products giving the position of ethylenic and probably acetylenic bonds in associated fatty acids. Further investigation of this twofold use of BF_3-MeOH in the ozonolysis of monoethylenic and polyethylenic fatty acids is projected.

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Titration of Sterol Double Bonds with Dibromopyridine Sulfate[1]

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ABSTRACT

Cis and *trans*-22-dehydrocholesteryl acetates and *cis* and *trans*-22-cholesten-3β-yl acetates were prepared and compared to Δ^{22}-phytosteryl acetates by titration with dibromopyridine sulfate. The cholesterol derivatives absorbed close to the theoretical quantity of bromine (1 Br_2 per double bond), whereas the Δ^{22}-C_{24}-alkylated sterols consumed 0.14 to 0.23 Br_2 in excess of the calculated values. This excess is attributed to the formation of additional unsaturation during bromination. Δ^7 and $\Delta^{8(14)}$-sterols consume more than 2 and 3 moles Br_2, respectively, which indicates that at least one or two new double bonds are formed in these molecules during the bromination step.

INTRODUCTION

In recent papers, we described the titration of various sterols with dibromopyridine sulfate (1,2). Δ^5-Sterols such as cholesterol, sitosterol, dihydrobrassicasterol and their esters absorbed 1 Br_2 per mole, and stigmastanol used none, as expected. The $\Delta^{5,22}$-sterols, stigmasterol, brassicasterol, and their esters, consistently absorbed 2.15-2.20 Br_2 per mole. We attributed this to the anomalous behavior of the *trans*-Δ^{22} double bond in the side chain of these compounds. In this paper, we present results obtained with some other sterols, notably *cis* and *trans*-Δ^{22}-cholesterol derivatives.

EXPERIMENTAL PROCEDURES

General Methods

Optical rotations were measured with Perkin-Elmer 141 and Rudolph DP 0801 instruments. Melting points are corrected. The ratios of *cis* to *trans* in mixtures of 22-dehydrocholesteryl acetates were estimated by IR (970 cm^{-1}) and thin layer chromatography (TLC) (10% $AgNO_3$-silica gel, 50:50:1 $CHCl_3$-CCl_4-HOAc). Bromine titrations were performed at 2-3 C as described (1) except where noted in Table I. P.e. is petroleum ether, b.p. 65-7 C (Skelly solve B).

Sterols

The acetates of sitosterol (1), brassicasterol (2), dihydrobrassicasterol (2), 7-stigmasten-3β-ol (3), 7,22-stigmastadien-3β-ol (3) and 7,9(11)-ergostadien-3β-yl benzoate (4) were available from earlier work. The acetates of cholesterol, stigmasterol, 7-cholesten-3β-ol, 7-ergosten-3β-ol, 7,22-ergostadien-3β-ol, and 8(14)-ergosten-3β-ol were prepared by conventional procedures (5). 5β-22-Stigmasten-3β-ol was a gift of H.J. Eyssen (6). Poriferasteryl acetate was prepared by the methods described by Sucrow et al. m.p. 148 C, $[\alpha]_D$-55.5°, lit (7) m.p. 146-8 C, $[\alpha]_D$-52.2°.

3β-Acetoxy-bisnor-5-cholen-22-al (2)

The aldehyde was prepared by modifications of McMorris' procedures (8). 3β-Acetoxy-bisnor-5-cholen-22-oic acid (1, 50 g, Steraloids, Inc. Wilton, NH) 100 ml thionyl chloride and 500 ml benzene were refluxed 1 hr, cooled and evaporated to dryness in vacuo. The residue was refluxed 1 hr with 100 g imidazole in 500 ml benzene, allowed to stand overnight, and the two phases evaporated to dryness. The residue was extracted with 1 liter. methanol and dried to yield 50 g imidazolid, m.p. 223.5-7 C, lit (8) 220-3 C. This was refluxed under N_2 in 700 ml tetrahydrofuran (THF) and reduced by the addition of 46 g LiAlH $(OtBu)_3$ in 500 ml THF to yield 43 g of an 80:20 mixture (gas liquid chromatography, GLC) of 2 and 3β-acetoxy-bisnor-5-cholen-22-ol (3) after conventional workup (8). Comparable reductions at room temperature gave lower yields of aldehyde. Separation of 2 and 3 was feasible (8), but not necessary for the subsequent Witting reaction; 2 m.p. 119-120.5 C (from acetone and p.e.), lit (8) 113-6 C, 3 m.p. 156-7.5 C (from acetone and ether), lit (9) 153-4 C.

5,22Z- and 5,22E- Cholestadien-3β-ylAcetates (4 and 5, respectively)

A solution of 320 g triphenylphosphine and 220 ml 1-bromo-3-methylbutane in 1500 ml xylene was refluxed 4 days. Solvent was decanted from the cooled mixture and the residue refluxed with dry THF containing a

[1]Arizona Agricultural Experiment Station Journal Article No. 2664.

TABLE I

Bromine Absorption by Sterol Double Bonds

Sterol type	Acetates of:	Br_2 per mole
Stanol	Bisnor-5α-cholan-22-oic acid	0.004,0.004
5-Monoene	Cholesterol	0.99,1.00
	Sitosterol	1.01,1.02
	Bisnor-5-cholen-22-oic acid	0.98,0.99
22-Monoene	22Z-Cholesten-3β-ol	1.02,1.03
	22E-Cholesten-3β-ol	1.03,1.04
	5β-22E-Stigmasten-3β-ol[1]	1.22,1.23
5,22-Diene	5,22Z-Cholestadien-3β-ol	2.01,2.01
	5,22E-Cholestadien-3β-ol	2.01,2.01
	Brassicasterol	2.17,2.19
	Poriferasterol	2.14,2.15
	Stigmasterol	2.17,2.20
		2.16[b],2.26[c]
7-Monoene	7-Cholesten-3β-ol	2.12,2.12
	7-Ergosten-3β-ol	2.13,2.16
	7-Stigmasten-3β-ol	2.03,2.09
7,9(11)-Diene	7,9(11)-Ergostadien-3β-ol[d]	2.08,2.10
7,22-Diene	7,22-Ergostadien-3β-ol	3,14,3.16
	7,22-Stigmastadien-3β-ol	2.99,3.01
8(14)-Monoene	8(14)-Ergosten-3β-ol	3.08,3.22

[a]Free sterol titrated
[b]−18 C
[c]28 C
[d]Benzoate titrated

little absolute ethanol. The salt was obtained on cooling in 53% yield, m.p. 154-6 C, lit (10) 158-9 C.

Butyl lithium (30 ml, 1.6 M in hexane) was added to a suspension of 21.9 g triphenyl-3-methylbutyl-phosphonium bromide in 300 ml dry p.e. and the mixture refluxed 5 min under N_2. A suspension of the **2-3** mixture (9 g) in 250 ml dry p.e. was quickly added to the hot solution and the resulting mixture stirred at 40-60 C 1 hr and at room temp overnight. The reaction was poured into water, the organic layer evaporated, the residue acetylated and crystallized from methanol to yield 5-6 g of a 25:75 mixture of **4** and **5**. The two were separated on 20% silver nitrate-silica gel columns (5 g/kg adsorbent) with 10:1 hexane-benzene and recrystallized from methanol: **4***(cis)* m.p. 116-7 C, $[\alpha]_D$-69.5°, lit (10) m.p. 115-6 C, $[\alpha]_D$-68°; **5**(*trans*) m.p. 128-9 C, $[\alpha]_D$-60.7' lit (10) m.p. 125-8 C, $[\alpha]_D$-61°. The methyl group nuclear magnetic resonances (NMR) of **4** and **5** corresponded to published values (10,11); in addition, there were significant differences in the absorptions of the side chain vinyl protons (Fig. 1).

5α-22Z- and 5α-22E-Cholesten-3β-yl Acetates (6 and 7, respectively)

Acid **1** was reduced with H_2-10% Pd/C-ethyl acetate to 3β-acetoxy-bisnor-5α-cholan-22-oic acid [m.p. 194-5.5 C, lit (12) 194 C], which was transformed by the methods described above to the *cis* and *trans* 5α-22-cholestenyl acetates: **6***(cis)* m.p. 104-5.5 C $[\alpha]_D$-20.8°; **7***(trans)* m.p. 105-6.5 C, $[\alpha]_D$-7.7°, lit (13) m.p. 104-5 C. The NMR spectra of the vinyl protons in the two compounds (Fig. 1) are analogous to those of **4** and **5** except for the absence of absorption at 5.37 (C_6 hydrogen) in **6** and **7**.

RESULTS AND DISCUSSION

The absorption of bromine from dibromo pyridine sulfate by the double bonds of various sterols is shown in Table I. Our earlier results (1,2) were confirmed; Δ^5 sterols consumed 1.00 ± 0.02 Br_2 per mole and $\Delta^{5,22}$ phytosterols 2.17 ± 0.03 Br_2 per mole. The influence of temperature on this overconsumption was small (Table I, stigmasteryl acetate). The data also show how substitution at C_{24} influences

bromine absorption. The four Δ^{22} and $\Delta^{5,22}$ compounds alkylated at C_{24} all consumed 0.14 to 0.23 moles of Br_2 in excess of calculated values, whereas the corresponding cholesterol derivatives (Δ^{22} *cis* or *trans*) consumed only 0.01 to 0.04 moles of excess Br_2 per mole of sterol.

The principal difference between these two classes of compounds which might explain this is the presence of an extra tertiary hydrogen atom, allylic to the double bond in C_{24}-alkyl sterols, which could participate in the development of some additional unsaturation during the bromination (Fig. 2A). The absence of a C_{24} tertiary hydrogen atom would then be the reason why Δ^{22} cholesterol derivatives (Fig. 2B) absorb nearly the theoretical amount of Br_2 per mole.

There are some justifications for this hypothesis. The loss of the proton on C_{25} from an intermediate carbonium ion to form a $\Delta^{24(25)}$ double bond is suggested to occur during phytosterol biosynthesis (14). In addition, the recovery of stigmasteryl acetate from its insoluble tetrabromide with zinc is only 78% (2) while the recovery of cholesterol from its 5,6-dibromide is at least 93% (15). This suggests that during the bromination of stigmasteryl acetate, compounds other than the 5,6;22,23-tetrabromide are formed.

Bromination of sterol double bonds in other

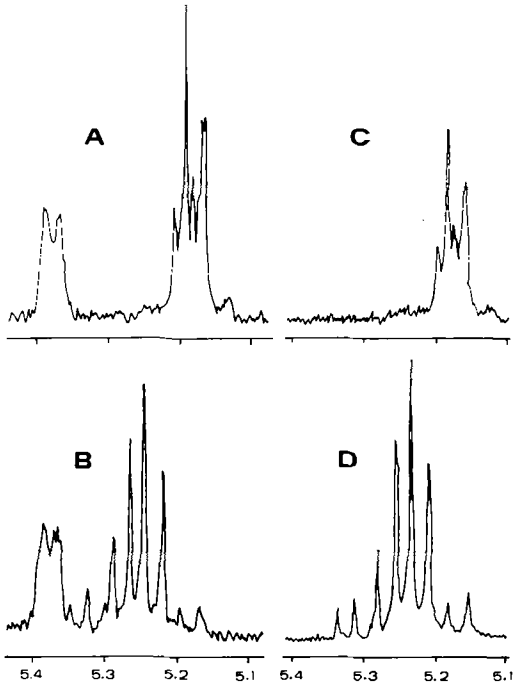

FIG. 1. Sections of the 270 MHZ spectra of compounds 4(A), 5(B), 6(C), and 7(D) showing the absorptions in the vinyl proton region. C_6 proton: δ = 5.37 (doublet); C_{22} and C_{23} protons: δ = 5.15 to 5.22 (multiplet) for the *cis*-Δ^{22} double bond. δ = 5.15 to 5.34 (octet) for the *trans*-Δ^{22} double bond.

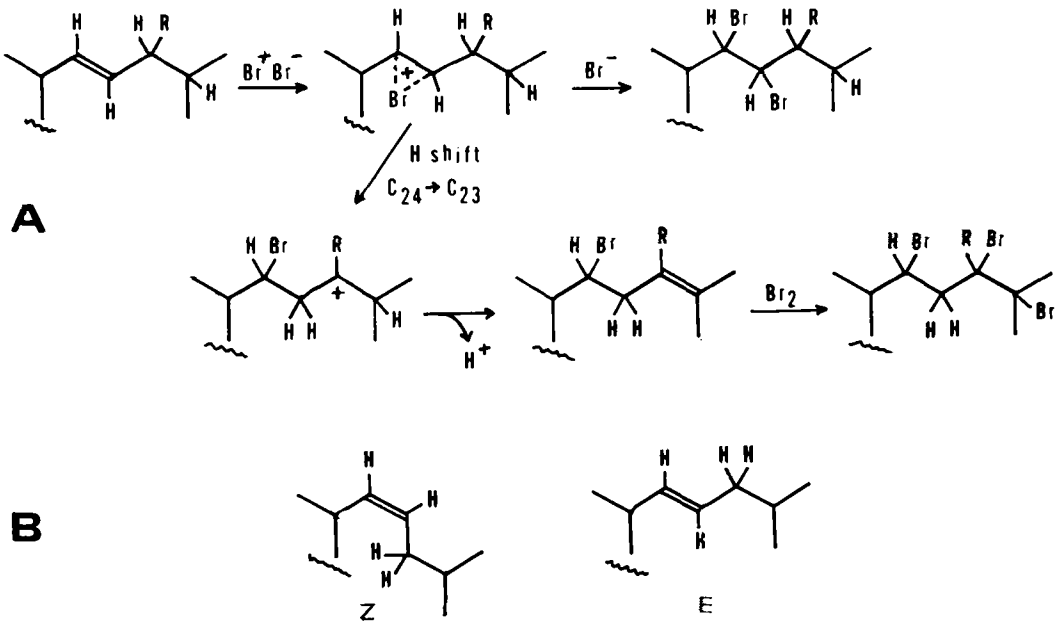

FIG. 2. A-Possible bromination pathways of C_{24}-alkylated (R = methyl or ethyl) sterols. B-Side chain of Δ^{22}-unsaturated cholesterols: Z = *cis*, E = *trans*.

positions gave widely varying results. Δ^7-Sterols consumed more than 2 Br_2 per mole, so an additional double bond must form during the bromination step. Zinc debromination of the reaction product followed by TLC and UV analysis showed the formation of 7,9(11)- and other dienes in these cases. Authentic 7,9(11)-ergostadienyl benzoate, however, absorbed only a small excess of Br_2 over the theoretical value of 2 Br_2 per mole.

During the bromination of 8(14)-ergostenyl acetate, two new double bonds must have formed to account for the 3+ Br_2 consumed per mole of this sterol. TLC of the products after zinc debromination showed complete absence of starting material with numerous very low R_f spots.

The analysis of sterols by this method of titration is, therefore, valid for stanols (0 Br_2/mole), Δ^5-stenols (1 Br_2/mole) and Δ^{22} or $\Delta^{5,22}$-cholesterol derivatives. The sterols we tested that have alkylation at C_{24} allylic to the Δ^{22} double bond or unsaturation at Δ^7 or $\Delta^{8(14)}$ consumed bromine in excess of the calculated values.

The Δ^{22}-cholestenols have not been prepared before, although the *trans* compound was reported once as a constituent of sponges (13). Our synthesis paralled that of the known $\Delta^{5,22}$ derivatives and the spectroscopic and chromatographic properties of the 22Z and 22E isomers were comparable to those of the 22-dehydrocholesterols.

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Changes in Rat Heart Phospholipid Composition After Rapeseed Oil Feeding

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ABSTRACT

The influence of long duration rapeseed oil feeding with high or low levels of erucic acid has been investigated on rat heart phospholipids. The rats treated for 20 wk with rapeseed oil containing 46.2% erucic acid showed a twofold increase in the sphingomyelin content of the heart. Treatment with primor rapeseed oil (3.7% erucic acid) for 20 wk did not modify phospholipid composition of rat heart. The fatty acid patterns of phosphatidylethanolamine and phosphatidylcholine were slightly influenced by the high erucic rapeseed oil; eicosenoic acid was incorporated preferentially into position one, but erucic acid showed a random distribution in both. After high erucic rapeseed oil feeding, 22:1 was incorporated into cardiolipin (5.6%) and sphingomyelin (10.5%). The incorporation of 22:1 into sphingomyelin was associated with an increase of the percentage of 24:1 (14.6%) and a decrease of saturated long chain fatty acid (22:0, 24:0) percentages. Primor rapeseed oil caused a slight increase of 24:1 and a decrease of 22:0 and 24:0 in rat heart sphingomyelin. As cardiolipin is localized in the inner membrane of mitochondria and sphingomyelin in plasma and microsomal membranes, the acyl-moiety alterations of both phospholipids might be correlated to the pathological lesions of rat heart after a long duration of rapeseed oil feeding.

INTRODUCTION

The pathological lesions of myocardium in rats after dietary rapeseed oil (RSO) have been well established by several investigators (1-4). Fatty acid infiltration was found in rat heart after feeding RSO or trierucin (5) for only 2 wk. Later on, RSO induced myocardial necrosis and fibrosis which appeared within 16 to 24 wk.

More recently, the comparative effect of RSO containing high or low levels of erucic (*cis*-13-docosenoic) acid (22:1) established the cardio-pathogenic effect of RSO containing a low level of erucic acid (2). The low erucic acid RSO (canbra, primor) did not produce the early cardiac lipidosis but caused the lesions later (6). Considerable attention has been given to the origin of cardiac pathological lesions obtained with these different RSOs. There is actual evidence that the early cardiac lipidosis is partly related to a slower mitochondrial oxidation of erucyl-CoA (7). Nevertheless, the origins of myocardial necrosis and fibrosis are still uncertain.

Kramer (8) studied the changes in liver lipid composition of rats on a 16 wk diet containing RSO differing in their erucic acid content. Erucic and eicosenoic (20:1) acids were incorporated into all lipid classes and the amount was proportional to that found in the dietary oil. Blomstrand and Svensson (9) investigated the incorporation of erucic acid in phospholipids of rat heart mitochondria, and found a specific affinity of erucic acid to cardiolipin after feeding RSO for 10 days. The authors suggested that erucic acid might influence the

TABLE I

Fatty Acid Composition of Dietary Oils

Fatty acid	Peanut oil (PNO)	Rapeseed oil (RSO)	Primor rapeseed oil (PRO)
16:0	10.1	3.4	5.1
16:1	0.3	0.3	0.3
18:0	3.9	1.0	1.7
18:1	52.5	14.6	55.7
18:2	27.9	14.1	20.5
18:3	-	8.0	11.1
20:0	1.6	1.0	-
20:1	1.1	11.0	1.9
22:0	2.6	0.4	-
22:1	-	46.2	3.7

TABLE II

Phospholipid Composition of Control and Treated Rat Hearts
(μmoles/g of wet tissue)

	Cardiolipin	Phosphatidylethanolamine	Phosphatidylcholine	Sphingomyelin
PNO[a]	2.8 ± 0.17[b]	9.3 ± 0.33	11.4 ± 0.32	0.8 ± 0.08
RSO[a]	3.1 ± 0.13	8.0 ± 0.25[c]	11.4 ± 0.22	1.5 ± 0.11[d]
PRO[a]	4.0 ± 0.26[c]	9.3 ± 0.58	13.3 ± 0.48	1.0 ± 1.1

[a]PNO: peanut oil, RSO: rapeseed oil, PRO: primor rapeseed oil.
[b]Mean ± standard error of the mean of sixteen pairs of rats.
[c]$P<0.05$.
[d]$P<0.001$.

normal function of the inner membrane of heart mitochondria in this way.

The phospholipid composition and their fatty acid patterns have not been previously investigated in rat heart, after dietary RSO with different levels of erucic acid has been given for several weeks. Moreover, sphingomyelin had never been examined in such experiments. In order to do so, we analyzed different classes of rat heart phospholipids [phosphatidylethanolamine (PE), phosphatidylcholine (PC), cardiolipin (CL), sphingomyelin (SP)], after feeding high or low erucic acid RSO for 20 wk.

MATERIALS AND METHODS

Animals and Diets

Three groups of 32 male, 4 wk old, Wistar rats were fed a diet containing 30 cal percent of fat for 20 wk. The control group was fed peanut oil containing no erucic acid. The remaining two groups were fed RSO with different erucic acid contents. High erucic (46.2%) RSO and low erucic (3.7%) primor RSO were the only source of fat in the diet (Table I).

Preparation of Total Lipid Extracts

After the animals were killed, the hearts of two rats were immediately removed, chilled in ice cold saline, cut into small pieces, washed and homogenized in a glass teflon homogenizer. Total lipids were extracted by the procedure of Folch et al. (10). The total lipid extract was stored in chloroform-BHT at -20 C for a maximum of 1 mo.

Phospholipid Measurements

An aliquot (about 15 μg of lipid phosphorus) was used to separate phospholipids by thin layer chromatography (TLC). After spotting, constant humidity of plates was obtained before migration by exposure to 9% H_2SO_4 for 15 min in a TLC chamber (Camag). PC, PE, and SP were separated on Silica Gel G (Merck) with chloroform-methanol-7 N NH_4OH (115:45:7.5 v/v) and CL with chloroform-methanol-acetic acid-acetone-water (100:20:20:40:10 v/v). After migration, the plates were air dried for a few minutes, then sprayed with rhodamine 6 G (10 mg in 100 ml of methanol) and the spots were identified under UV light. The different spots were scraped and the phospholipids were eluted. After evaporation to dryness, the concentration of phospholipid was measured by a colorimetric method using ammonium-molybdate (11). Results were expressed in μmoles/g of wet tissue.

Phospholipid Separation Using Silicic Acid Chromatography

Phospholipid separation on silicic acid column was carried out as described previously (12). Ca. 40 mg total lipids dissolved in chloroform were transferred to the column. After elution of neutral lipids with chloroform (10 volumes) and glycolipids with acetone (40 volumes), four fractions were obtained using the following elution mixtures: (A) chloroform-methanol 95:5 (10 volumes); (B) chloroform-methanol 80:20 (20 volumes); (C) chloroform-methanol 50:50 (20 volumes); and (D) methanol (20 volumes). The fraction (A) contained CL; the fraction (B), mainly PE; the fraction (C), PC; and the fraction (D), SP. The predominant phospholipid of each fraction was isolated by TLC. The plates were prepared as described and the phospholipid separated by chloroform-methanol-7 N NH_4OH (115:45:7.5 v/v). Before isolation by TLC, the fraction (D) containing SP was treated by the alkaline methanolysis procedure (13) in order to exclude phosphatidylinositol and lysophosphatidylcholine. After migration, the plates were sprayed with rhodamine 6 G and the spots were identified under UV light.

Fatty Acid Analysis

The positioning of the fatty acids in PE and

TABLE III

Relative Composition of Total Fatty Acids (moles %) of Cardiolipin (CL), Phosphatidylethanolamine (PE), Phosphatidylcholine (PC), and Sphingomyelin (SP) in Control and Treated Rat Hearts

Fatty acid	CL			PE			PC			SP		
	PNO[a]	RSO[a]	PRO[a]	PNO	RSO	PRO	PNO	RSO	PRO	PNO	RSO	PRO
16:0	4.0 ± 1.7[b]	2.5 ± 1.3	2.2 ± 1.8	7.5 ± 2.4	6.2 ± 1.0	7.4 ± 2.0	14.1 ± 2.9	11.6 ± 2.8	14.8 ± 2.9	13.7 ± 3.3	17.4 ± 3.6	21.2 ± 5.2
16:1	1.7 ± 0.7	0.5 ± 0.3	0.4 ± 0.2	0.8 ± 0.6	0.3 ± 0.3	0.6 ± 0.3	2.1 ± 2.1	1.2 ± 0.5	0.9 ± 0.8	0.9 ± 1.0	1.1 ± 1.2	1.7 ± 2.4
18:0	3.9 ± 1.6	2.6 ± 1.6	2.8 ± 2.3	25.9 ± 5.3	28.8 ± 6.5	28.4 ± 2.9	31.6 ± 3.9	28.6 ± 7.6	30.5 ± 2.4	12.6 ± 0.9	9.8 ± 1.5	20.1 ± 3.1
18:1	9.9 ± 1.7	6.0 ± 1.4	8.8 ± 2.0	10.8 ± 3.4	13.4 ± 6.0	10.9 ± 2.3	10.3 ± 2.7	9.3 ± 2.2	12.9 ± 3.4	3.1 ± 1.1	3.3 ± 2.8	3.9 ± 2.4
18:2	73.2 ± 6.1	79.5 ± 4.3	82.6 ± 3.9	5.1 ± 1.0	7.2 ± 1.5	5.6 ± 1.2	7.3 ± 1.1	12.7 ± 3.7	8.2 ± 2.0	-	0.5 ± 0.5	1.0 ± 1.5
20:0	0.6 ± 1.2	0.6 ± 1.1	-	-	-	-	-	-	-	17.3 ± 1.1	17.0 ± 5.6	23.1 ± 2.9
20:1	-	0.6 ± 1.0	-	-	1.7 ± 0.4	0.1 ± 0.2	-	1.4 ± 0.3	-	-	0.4 ± 0.7	-
20:4	3.6 ± 0.5	0.9 ± 1.3	1.6 ± 1.1	29.8 ± 5.1	21.7 ± 3.3	23.0 ± 2.6	32.0 ± 7.2	29.2 ± 5.5	27.6 ± 4.4	-	-	-
22:0	-	-	-	-	0.6 ± 1.2	-	-	0.4 ± 0.5	-	38.6 ± 4.1	22.1 ± 2.9	18.2 ± 4.0
22:1	-	5.6 ± 2.8	0.5 ± 0.7	-	1.2 ± 0.4	0.3 ± 0.3	-	2.4 ± 2.7	0.1 ± 0.1	-	10.5 ± 2.5	1.0 ± 0.8
22:5	0.3 ± 0.3	-	-	3.8 ± 1.2	4.0 ± 1.1	3.5 ± 0.9	1.1 ± 0.5	1.5 ± 0.4	2.0 ± 1.1	-	-	-
22:6	2.8 ± 1.0	1.2 ± 0.9	1.1 ± 1.5	16.3 ± 5.0	14.9 ± 4.1	20.2 ± 5.9	1.6 ± 0.9	1.7 ± 0.4	3.0 ± 1.5	-	-	-
24:0	-	-	-	-	-	-	-	-	-	10.3 ± 2.2	3.3 ± 0.5	3.0 ± 1.4
24:1	-	-	-	-	-	-	-	-	-	3.5 ± 2.2	14.6 ± 1.4	6.8 ± 3.4

[a]PNO: peanut oil, RSO: rapeseed oil, PRO: primor rapeseed oil.

[b]Mean ± standard deviation of sixteen pairs of rats.

TABLE IV

Relative Distribution of Total Fatty Acids (moles %) Esterified to the 1 and 2 Position of Phosphatidylethanolamine (PE) and Phosphatidylcholine (PC) in Control and Treated Rat Hearts

Fatty acid	PC						PE					
	PNO[a]		RSO[a]		PRO[a]		PNO		RSO		PRO	
	1	2	1	2	1	2	1	2	1	2	1	2
16:0	19.0 ± 2.9[b]	6.2 ± 2.2	22.7 ± 8.8	10.2 ± 3.2	24.3 ± 6.5	10.0 ± 3.9	13.2 ± 4.8	2.6 ± 0.6	13.5 ± 3.5	3.5 ± 1.7	13.9 ± 1.9	3.5 ± 1.2
16:1	1.3 ± 1.7	0.8 ± 0.9	0.6 ± 0.8	1.4 ± 1.7	3.0 ± 3.4	0.9 ± 0.5	1.6 ± 1.2	0.6 ± 0.3	1.3 ± 1.3	0.4 ± 0.3	1.5 ± 0.9	0.5 ± 0.3
18:0	66.9 ± 2.1	1.6 ± 0.8	64.6 ± 9.8	2.3 ± 1.6	58.0 ± 9.9	5.1 ± 4.3	62.8 ± 3.2	2.1 ± 0.7	57.8 ± 6.7	3.1 ± 1.9	59.9 ± 3.7	3.3 ± 1.9
18:1	8.5 ± 2.0	9.8 ± 3.2	5.6 ± 2.4	11.9 ± 2.3	9.5 ± 2.4	15.6 ± 5.3	18.0 ± 4.5	6.6 ± 0.8	17.9 ± 4.7	6.3 ± 1.3	14.9 ± 4.2	8.2 ± 3.4
18:2	4.3 ± 1.9	8.0 ± 1.8	4.9 ± 1.3	16.0 ± 2.1	5.2 ± 2.3	10.4 ± 2.7	4.4 ± 1.7	5.3 ± 0.7	4.7 ± 1.3	7.7 ± 1.9	7.2 ± 5.7	7.0 ± 2.3
20:1	-	-	1.5 ± 0.9	0.5 ± 0.3	-	-	-	-	2.0 ± 1.1	1.2 ± 0.4	0.7 ± 1.0	-
20:4	-	68.2 ± 7.3	-	52.0 ± 6.3	-	49.3 ± 7.5	-	48.8 ± 2.4	-	41.3 ± 8.0	-	37.0 ± 4.3
22:0	-	-	-	-	-	0.1 ± 0.2	-	-	0.9 ± 2.6	-	1.4 ± 1.4	-
22:1	-	-	0.1 ± 0.2	0.8 ± 0.4	-	0.4 ± 0.3	-	-	1.9 ± 3.0	1.0 ± 0.3	0.5 ± 0.6	0.3 ± 0.2
22:5	-	2.2 ± 0.7	-	2.0 ± 0.6	-	3.1 ± 0.8	-	5.5 ± 0.9	-	7.4 ± 1.6	-	6.1 ± 1.4
22:6	-	3.2 ± 1.0	-	2.9 ± 1.3	-	5.1 ± 2.4	-	28.5 ± 3.6	-	28.1 ± 5.8	-	34.1 ± 7.8

[a]PNO: peanut oil, RSO: rapeseed oil, PRO: primor rapeseed oil.
[b]Mean ± standard deviation of sixteen pairs of rats.

PC was determined by hydrolysis with phospholipase A_2 (Crotalus adamenteus). The substrate was introduced into buffered enzyme solution in diethyl-ether (1 mg of lipid per ml of ether). The incubations were continued for 90 min at 30 C with stirring. The lysophospholipids formed and fatty acids liberated were separated by TLC using chloroform-methanol-7 N NH_4OH (115:45:7.5 v/v) as developer. The relative distribution of fatty acids in PE, PC, CL, and SP was investigated as follows. The methyl esters of fatty acids were obtained by transesterification of lipids with methanol-H_2SO_4 at 70 C (14) after 2 hr for CL, PE, and PC or 24 hr for SP. After extraction by heptane, the methyl esters were purified on TLC using benzene. The fatty acid methyl esters were separated and identified by gas liquid chromatography (GLC) using a capillary column of carbowax 20 M (50 m) at 200 C. The different peak areas were calculated by integration (Spectraphysics). The results were expressed in moles percent.

RESULTS AND DISCUSSION

Phospholipid measurements showed a striking change in the distribution of the cardiac phospholipids in which SP increased twofold ($P<0.001$) in the rats fed high erucic RSO for 20 wk (Table II). Our results are in agreement with Beare-Rogers (15) who found a threefold increase of SP in similar conditions with high erucic RSO. In our experiments, primor RSO containing low levels of 22:1 did not induce an increase of SP after 20 wk.

The influence of RSO diet (containing 46% erucic acid) on the fatty acid pattern in PC from rat heart is shown in Table III. The fatty acids of PC and PE were slightly modified by the high erucic RSO diet. Erucic acid was incorporated into PE (1.2%) and PC (2.4%). Eicosenoic acid was found in both phospholipids (PE 1.7% and PC 1.4%). The presence of 20:1 could be related to dietary intake (11%) although the contribution of β-oxidation of 22:1 cannot be eliminated (16). The positioning of the fatty acids in PE and PC (Table IV) showed a random distribution of 22:1 between both positions. Nevertheless, 20:1 was incorporated preferentially into position one as described by Kramer in liver phospholipids of rats fed on high erucic RSO (8). With primor RSO containing a low level of 22:1, very small changes were observed in the fatty acid patterns of PE and PC (Tables III and IV).

In fact, erucic acid seems to have a specific affinity for CL and SP. The isolated CL from hearts of rats fed with high erucic RSO was

found to contain 5.6% of 22:1. Eicosenoic acid was also present at a lower level (0.6%) in CL. Our results confirmed the incorporation of erucic acid into CL already described by Blomstrand and Svensson (9). Nevertheless, we did not obtain any difference in 18:2 percentages as compared with their results. With primor RSO, no significant changes were found in the fatty acid distribution of CL.

CL is synthesized exclusively in the inner mitochondrial membrane (17) and may be required for its integrity. Moreover, there is now good evidence that in CL, a fatty acid exchange (acylation-deacylation) occurs and that acyl-moieties exhibit a much faster turnover rate than the remaining molecular components (18). The independent turn-over of acyl groups could explain the incorporation of 22:1 into this mitochondrial phospholipid after RSO feeding.

Following the incorporation of erucic acid into CL, the mitochondrial function could be altered, this phospholipid being tightly bound to cytochrome oxidase (19) and ATP-ase (20). Moreover, the monounsaturated erucic acid has physical properties like those of saturated fatty acids and it has been shown that a certain proportion of unsaturated fatty acids are necessary to maintain mitochondrial oxidative phosphorylation (21). The presence of 22:1 into CL might modify its physicochemical state and determine a specific inhibitory effect upon mitochondrial function.

The most striking change, never before established, induced by high or low erucic RSO was observed in the fatty acid pattern of SP (Table III). After high erucic RSO feeding, 22:1 was incorporated to a large extent in SP (10.5%) and there was an increase in the percentage of nervonic acid (from 3.5% to 14.6%). Saturated fatty acids (22:0 and 24:0) of SP were decreased at the same time; behenic acid (22:0) content decreased from 38.6% in controls to 22.1% in treated rats and lignoceric acid (24:0) content from 10.3% to 3.3%. The ratio (C_{22}/C_{24}) decreased from 2.8 to 1.8. These modifications of SP might be related to its increased concentration observed at the same time. Primor RSO induced minor modifications in the fatty acid pattern of SP and it caused a decrease of 22:0 and 24:0 with an increase of 24:1 and 18:0, while 22:1 was incorporated to a small extent.

The increase of nervonic acid in SP is related to erucic acid incorporation and its intramyocardial elongation (16). Also, long chain saturated fatty acids being specifically used for the biosynthesis of SP might explain the specific incorporation of 22:1 into this phospholipid membrane structure.

The elevated concentration of cardiac SP and the modification of its fatty acid pattern seems to indicate a degenerative process in the heart cell membranes. This could be related to necrosis and fibrosis occurring in the hearts of rats fed RSO. SP is found in plasma and microsomal membranes and plays an important role in the cellular structure as a whole (22). Moreover, the increase of SP content might reflect a secondary lysosomal storage disorder (23).

In conclusion, our study shows incorporation of erucic acid into rat heart CL and SP after long duration RSO feeding. Nevertheless, the mechanism by which phospholipid modifications could be correlated to pathological lesions, observed by many authors in the same conditions, remains to be established.

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Positional Isomers of Unsaturated Fatty Acids in Rat Liver Lipids

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ABSTRACT

The fatty acids of liver lipids from rats raised on a fat free diet from the 30th to the 90th day after birth were analyzed with special regard to the detection of positional isomers of mono-, di-, tri-, and tetraenoic fatty acids. The methyl esters obtained after transesterification of total lipids were separated by argentation chromatography into five fractions: I saturated, II monoenoic, III dienoic, IV dienoic nonmethylene interrupted, V tri- and tetraenoic fatty acid esters. After hydroxylation of the double bonds with osmium tetroxide, the analysis of the poly-O-trimethylsilyl derivatives by gas liquid chromatography on S.C.O.T. columns combined with mass spectrometry revealed the presence of 19 monoenoic, 15 dienoic, and 9 trienoic as well as 3 tetraenoic fatty acid isomers including the normally occurring representatives of the (n-3), (n-6), (n-7), and (n-9) fatty acid families. The majority of the identified isomers can be coordinated to one of these families like 7-16:1; 11-20:1; 6,9-18:2; 8,11-20:2; 5,11-20:2; 5,8,11-20:3; 7,10,13-22:3 to the (n-9) family, 11-18:1; 13-20:1; 5,11-18:2; 7,13-20:2; 6,11-18:2; 6,9-16:2; 8,11-18:2; 10,13-20:2; 5,8,11-18:3; 7,10,13-20:3; 4,7,10,13-20:4 to the (n-7) family and 11,14-20:2; 5,11,14-20:3; 6,9,12-18:3; 8,11,14-20:3; 5,8,11,14-20:4; 7,10,13,16-22:4 to the (n-6) family. All these naturally occuring isomers can be placed into a network of desaturation and chain elongation steps which allows certain conclusions about the substrate specificity of the Δ6-, Δ5- and Δ4-desaturase systems. The great number of isomers found in the (n-7) family indicates that the members of this family are actively metabolized in partial essential fatty acid deficiency.

INTRODUCTION

In higher organisms, C_{16} and C_{18} fatty acids with one, two, or three double bonds are submitted to consecutive chain elongation and oxygen dependent desaturation steps. Since the second and further double bonds are introduced between the carboxyl group and the already existing one, normally separated by one methylene group, the position of the double bond next to the methyl end remains unchanged throughout all metabolic conversions, thus giving rise to the well known (n-9), (n-7), (n-6), and (n-3) fatty acid families (1-3). Arachidonic and docosahexaenoic acids as major end products of linoleic and linolenic acids in mammals can be produced by possibly several alternative pathways. The order of consecutive desaturation and chain elongation steps has up to now only in a few cases been determined and is still a matter of debate (4-8).

Differing fatty acid patterns in different cells indicate that fatty acids have a choice of a number of competing desaturation and elongation reactions and evidence has been produced that the biosynthesis of the polyunsaturated fatty acids is controlled by competition between different members of the fatty acid families for the same binding site on the desaturase system (9-11). These competitive mechanisms of regulation can be modulated by other environmental factors like temperature, pH, salt concentration, and dietetic manipulations (12,13). Using labelled precursors and analogs of the different fatty acid families in vitro as well as in vivo, data have been presented by workers from several laboratories concerning points of regulation for the Δ6- and Δ5-desaturase systems (14,15). Further experimental data indicate the presence of different chain elongation systems acting on saturated, mono- and polyenoic acids respectively (16).

Although the fatty acid composition of liver lipids has been frequently analyzed and the major components are well known (17-19), little information exists about minor constituents such as to be expected to arise as by-products of competitive mechanisms. After the development of refined methods for the separation and identification of unsaturated fatty acids using capillary gas liquid chromatography (GLC) in combination with mass spectrometry (MS) (20-22), the present investigation was started with the aim of identifying by-products of the fatty acid metabolism. Furthermore, the

TABLE I

Distribution of Major Components of Fatty Acids from Total Liver Lipids of Rats Raised on a Fat Free Diet and of Fractions I to V Obtained after Preparative Argentation Chromatography

Fatty acid component	Total liver lipids	Fraction I	II	III	IV	V
16:0	20.2	63.8				
16:1	7.3		16.3	5.8		
18:0	20.6	35.0				
18:1	18.1		83.3	3.3		
18:2	4.4			84.7	82.0	
20:1	0.1					
20:2	0.2			3.9		
20:3(n-9)	4.6				11.4	15.6
20:3(n-6)	1.1				2.2	
20:4	14.3					73.2
22:4	2.6					2.2
22:6	4.7					7.9

occurrence of such isomers should give some insight into substrate specificity and structural requirements of the binding site of the different desaturase systems. With the aim of increasing the endogenous Δ6- and Δ9-desaturase activity and simultaneously the yield of certain isomers, the animals were kept on a fat free diet for 9 wk (23).

MATERIALS AND METHODS

Materials

All solvents and reagents were of analytical grade unless otherwise stated. Osmium tetroxide (Degussa, Hanau, GFR) was purified by sublimation. Solvents were redistilled over 1 m columns before use; 0.1% (w/v) 2.6-di-tert.-butyl-4-hydroxytoluene (BHT) was added to all solvents used for the extraction or chromatographic purification of lipids in order to prevent autoxidation of polyenoic fatty acids.

Animals and Diets

Female wistar rats of own breed were kept on a fat free diet from the 30th to 93rd day after birth. The diet (Altromin Sonderdiät C 1006, 4910 Lage, GFR) was composed of 22% casein, 56% starch, 10% sucrose, 4% cellulose powder, 6% minerals and trace elements, and 2% vitamin mixture. The diet contained 0.3% lipid of dry weight as determined after twofold extraction with hot chloroform/methanol 2:1 for 3 hr. It contained no unsaturated fatty acids as controlled by combined GLC-MS. Control animals were kept on normal laboratory chow (Altromin). Livers were removed from the animals anaesthetized with ether and immediately frozen in liquid nitrogen.

Lipid Extraction

The livers were homogenized with 20 vol (v/w) chloroform/methanol 2:1 in a Potter-Elvehjem homogenizer. After filtration, the residue was extracted under reflux in N_2 atmosphere with $CHCl_3$/MeOH. The combined extracts were washed with 1/5 vol water and twice with theoretical upper phase (24).

Analytical Procedures

Rapid thin layer chromatography (TLC) for densitometric determinations (25) or monitoring of reactions was carried out on 7.5 x 2.5 cm microscope slides coated with silica gel Camag DO (Buchs, Switzerland). The spots were visualized by spraying with 10% sulfuric acid (v/v) followed by charring on hot plates of 100 C and 200 C. Quantitative determination of neutral lipids followed the modified procedures of van Gent (26); phospholipid distribution was assayed after two dimensional TLC according to Debuch (27).

Total lipids were transesterified with 5% anhydrous HCl in methanol at room temperature. The reaction was complete after 11 hr as monitored by TLC. After evaporation under N_2 in a rotatory still, traces of free fatty acids were esterified by the addition of etheral diazomethane. The methyl esters of fatty acids were separated from cholesterol and other saponification products by elution with chloroform/n-hexane, 1:4, from a silica gel column (Silicagel HR 60, Merck, Darmstadt, GFR). The separation of the fatty acid methyl esters on $AgNO_3$ impregnated silica gel according to number, position, and configuration of double bonds has already been described in detail as well as procedures for hydroxylation with OsO_4, silylation and conditions of GLC-MS analysis (21). Other conditions are given in the legend to Figure 2.

RESULTS

After being for 60 days on a fat free diet, the animals started to show typical signs of essential fatty acid deficiency.

The pooled methyl esters of the fatty acids from liver lipids were separated on silica gel impregnated with silver nitrate into five fractions according to the number of double bonds. The composition of the fractions obtained is given in Table I for the main components. Fraction IV still contains predominantly 9,12-octadecadienoic acid, probably due to overloading of the plates.

After hydroxylation with OsO_4 and silylation, the derivatives of the fatty acids from fraction II-V were analyzed by combined GLC-MS. The substitution at the place of the original double bond aggravates the difference between positional isomers and facilitates the gas chromatographic separation. The clearcut mass spectrometric fragmentation of the trimethylsilyloxy (OTMS)-derivatives of unsaturated fatty acids under low energy electron impact permits a rapid and unequivocal identification also of hitherto unknown unsaturated fatty acids. Primary ions are almost exclusively formed by splitting of the C-C bond between the vicinal OTMS groups. Primary ions containing five or more OTMS groups favor a stabilization after splitting off three or more trimethylsilanol (HOTMS) groups possibly with concomitant formation of an aromatic ring system. Loss of four and five HOTMS groups is also observed with primary ions carrying seven OTMS substituents. The loss of HOTMS groups is usually accompanied by the occurrence of intense metastable ions. Fragments derived from the carboxyl end can be recognized by a group of ions produced by rearrangement and the addition of one TMS group furnishing ions like A+73, C+73, and F+73. In fragments carrying the same number of OTMS groups, those which are derived from the methyl end are mostly more intense than those from the carboxyl end. The fragments A+102, A+102-90 and N+102, N+102-90 are of minor intensity (28,29). Fatty acids of the same family all produce the same fragment N.

The distance between the original double bonds is indicated by fragment E (Scheme I). Although no molecular ion could be obtained in any of these poly-OTMS derivatives, M can be calculated by the addition of all corresponding primary ions like A+B, C+D, F+G, and H+N. Thus, a rapid and sensitive method is at hand which allows the analysis of very complex mixtures of isomeric unsaturated fatty acids and the interpretation of mass spectra obtained from unresolved peaks.

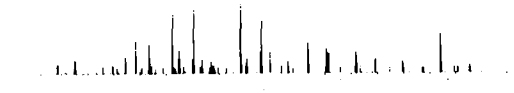


FIG. 1. 20 eV Mass spectrum of 1-Trimethylsilyl-5,6-8,9, 11,12-14,15-octa-O-trimethylsilyl-eicosanoate.

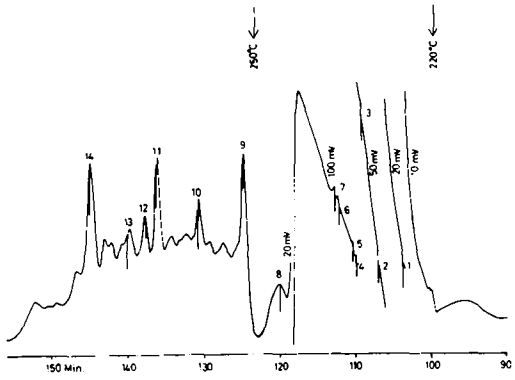
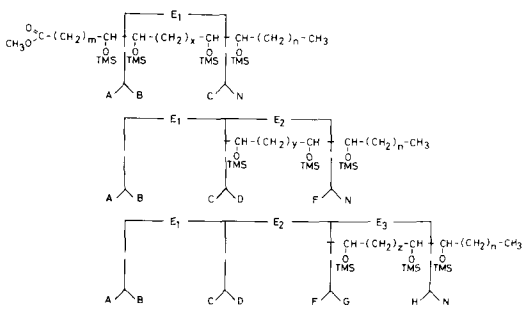


FIG. 2. Part of the gas chromatogram of poly-trimethylsilyloxy (OMTS) derivatives of fraction IV from preparative thin layer chromatography on silica gel impregnated with silver nitrate. Column: 50 ft x 0.02 in. S.C.O.T. SE 30 plus 50 ft x 0.02 in. S.C.O.T. Dexsil GC 300 (Perkin Elmer). Oven temp.: as indicated in the diagram. Detector: total ion current at 20 eV. Attenuation: mV as given in the diagram. Injection port temp.: 280 C. Carrier gas: He, ca 4ml/min. Compounds identified in this run: (1) Methyl-8,9,11,12-tetra-OTMS-octadecanoate, (2) Methyl-5,6,8,9-tetra-OTMS-octadecanoate, (3-7) main component Methyl-9,10,12,13-tetra-OTMS-octadecanoate, (8) Methyl-5,6,11,12-and Methyl-6,7,12,13-tetra-OTMS-octadecanoate, (9) paraffin, (10) not identified, (11) Methyl-8,9,11,12-tetra-OTMS-eicosanoate, (12) Methyl-10,11,13,14-tetra-OTMS-eicosanoate, (13) paraffin, (14) Methyl-5,6,11,12-and Methyl-7,8,13,14-tetra-OTMS-eicosanoate.

Exceptions from this rule have so far only been observed with 2. 3-di-OTMS derivatives of fatty acid methyl esters (30).

Another peculiarity is shown by the OTMS derivatives of 5,8,11-20:3 and 5,8,11,14-20:4. During derivatization, these fatty acids tend to form a TMS-ester probably by rearrangement. These derivatives bearing TMS instead of methyl at the carboxyl group show larger retention times in GLC and all ions containing the carboxyl group are shifted to 58 amu higher values. The main fragmentation pattern remains



SCHEME I. Fragmentation pattern of poly-OTMS derivatives of di-, tri- and tetraenoic fatty acid methyl esters.

unchanged, as exemplified by the mass spectrum of TMS-5,6-8,9-11,12-14,15-octa-OTMS-eicosanoate (Fig. 1).

A typical gas chromatographic separation of fraction IV is presented in Figure 2. Mass spectrometrically identified compounds of this run are listed in the legend to Figure 2.

The fatty acids identified as poly-OTMS derivatives including also the principal members of the major four fatty acid families are listed in Table II. The mass spectrometric data, necessary for the identification of the poly-OTMS derivatives of the fatty acids listed in Table II, are presented in Table III.

DISCUSSION

With the exception of the uneven numbered fatty acids, most of the identified di-, tri-, and tetraenoic acids can be attributed to one of the known fatty acid families as outlined in Scheme II.

Metabolic pathways, which have already been demonstrated by other laboratories using labelled substrates are shown in solid lines. Chain elongation steps are indicated by vertical arrows, desaturation steps by horizontal ones. Hypothetical pathways, leading to the formation of isomeric enoic fatty acids are indicated by dotted lines. Penta- and hexaenoic acids are not included in the scheme. Due to the prevalence of 9-18:1 as a typical consequence of essential fatty acid deficiency, this acid serves as substrate for several enzymatic reactions: β-oxydation (7-16:1), chain elongation (11-20:1), and further desaturation (6,9-18:2). Also 11-20:1 can be further desaturated to 5,11-20:2 as already shown by Ullman and Sprecher using [3-^{14}C]-11-20:1 in feeding studies with rats or in vitro with rat liver micro-

TABLE II

Mass Spectrometrically Identified Fatty Acids from Fractions II to V

Chain length	Position of double bonds			
	Monoenoic	Dienoic	Trienoic	Tetraenoic
	Fatty acids			
14	9	–	–	–
15	9	–	–	–
16	6	6,9	–	–
	7			
	9			
	11			
	12			
17	6	–	–	–
	9			
	10			
	11			
18	2	4,7	5,8,11	–
	6	5,8	6,9,12	
	8	6,9	9,12,15	
	9	8,11		
	11	9,12		
	13	9,13		
		6,11		
		5,11		
		6,12		
19	–	–	5,8,11	–
20	11	8,11	5,8,11	5,8,11,14
	13	10,13	7,10,13	4,7,10,13
		11,14	8,11,14	
		5,11	5,11,14	
		7,13		
22	–	–	7,10,13	7,10,13,16

TABLE III

Relative Intensities in % of Major Fragments Obtained by Mass Spectrometric Analysis of the Trimethylsilyloxy (OTMS) Derivatives of the Polyenoic Fatty Acid Methyl Esters Listed in Table II

Fragments of OTMS Derivatives according to Scheme 1	Original fatty acid methyl esters and relative intensities of fragments																								
	6,9-16:2[a]	4,7-18:2	5,8-18:2[b]	6,9-18:2[a]	8,11-18:2[b]	9,12-18:2[b]	9,13-18:2	6,11-18:2[b]	5,11-18:2	6,12-18:2	8,11-20:2	10,13-20:2	11,14-20:2	5,11-20:2	7,13-20:2	5,8,11-18:3	6,9,12-18:3[ac]	9,12,15-18:3[cd]	5,8,11-19:3	5,8,11-20:3	7,10,13-20:3	8,11,14-20:3	5,11,14-20:3	7,10,13-22:3	5,8,11,14-20:4	4,7,10,13-20:4	7,10,13,16-22:4
A	28	6	48	31	20	22	57	6	100	69	24	46	19	100	100	40	20	100	44	33	13	15	67	11	40	12	6
A+73	3	46		4	5	3	7		33	28	6	3	2	36	70	8	4		6	3		2	24	5	5		
B	6	2	6	4	7	6	9	5	2	12	6	6	8	9	11	1	4					3	7	5	2		3
B-90	100	100	88	88	100	100	100	100	10	50	100	100	100	14	61	3	2	7	5	1		2		3	1		
B-180	6	55		2	20	4		2	12	40	4	2	3	21	46	25	26	95	28	21	31	27		21	3		3
B-270											3					9	8	8	5	8	8	10	6	8	9		10
B-360																	2		7	9		1	5		2	7	6
B-450																									2	15	
B-540																									1		
C	4		5	4	3	2	4	3	15	3	4	2	4	16	27	11	10	7	15	10	13	7	4	10	13		16
C+73																							7	5			
C-90	60	32	100	100	85	85	61	84	11	100	91	91	72	16	52	84	84	95	100	68		82	7	82	85	82	90
C-180	7	20	12	7	4	4		4	17	6	5	2		26	32	5	5	8	6	6	12	3	10	2	3	24	
C-270							25		10		5	1			10											11	18
D																11	12		16	11	24	12	7	13	8		20
D-90																100	100	100	100	100	100	100	100	100	3		9
D-180																	1	6		1		1			39	49	
D-270																			18						9	12	8
D-360																									1		8
F																3	3			2		1		3	5		6
F-90																2	2		5	1	23	1	38		3	23	16
F-180																26	22	10	34	24	11	21		18	2		26
F-270																16	11	5	17	12		10	5	8	10	8	11
F-360																4	4		7	4		2	5		4	10	
F-450																			4			1			1		
G																									18	18	11
G-90																									100	100	100
G-180																									1		
H																									2		
H-90																									1	31	
H-180																									2	19	
H-270																									9	22	14
H-360																										9	8
N	13	7	13	17	12	14	9	2	37	35	17	14	28	47	73	14	13	16	18	12	11	10	16	9	11	23	16
E[e]	28	38	7	31	7	6	11	22	19	40	9	6	9	27	27	20	20	19	21	9	46	9		9	13	7	19
E_1																							5				
E_2																							10				

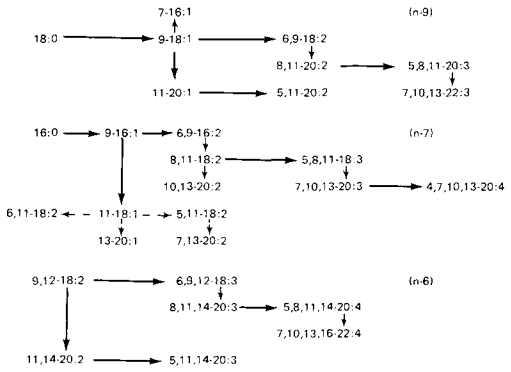
[a]In these spectra fragments A and E appear at the same mass number.

[b]Spectra of these acids derived from other sources have already been described (28).

[c]Spectra of these acids derived from other sources have already been described (29).

[d]With this derivative the fragments C and B-90 as well as A and D-90 appear at the same mass number.

[e]In these spectra $E_1 = E_2 = E_3$.



SCHEME II. Possible pathways for the biosynthesis of the fatty acids of the (n-9), (n-7) and (n-6) families.

somes (31).

In a similar way, 9-16:1 seems to become available for several metabolic conversions normally occupied by the fatty acids of the (n-6) family. The sequence 6,9-16:2; 8,11-18:2; 10,13-20:2 as well as 6,9-18:2; 8,11-20:2 are in accordance with the findings of Bernert and Sprecher (14). Besides desaturation of 9-16:1 to 6,9-16:2, chain elongation yields 11-18:1. This acid may then serve as a substrate for further desaturation as indicated by 6,11-18:2; 5,11-18:2; and 7,13-20:2. These results show that *cis*-vaccenic acid (11-18:1) is subjected to several metabolic reactions in rat liver during essential fatty acid deficiency.

Among the fatty acids of the (n-6) family, the occurence of 5,11,14-20:3 indicates that 11,14-20:2 can also serve as substrate for Δ5-desaturation as shown independently by Ullman and Sprecher (31) by feeding [1-^{14}C]-11,14-20:2 to rats raised on a fat deficient diet.

A number of fatty acids listed in Table II do not fit into any of the four fatty acid families. Among these, the odd numbered fatty acid 5,8,11-19:3 may be produced by the reaction sequence 9-17:1 → 6,9-17:2 → 8,11-19:2 → 5,8,11-19:3. Other even and odd numbered monoenoic acids not belonging to any of the families may be the products of α-oxidation. Four of the octadecadienoic acids listed in Table II are not contained in Scheme II. The (n-5) fatty acids may be genetically linked by the following reaction sequence 9-14:1 → 11-16:1 → 13-18:1 → 9,13-18:2. A similar Δ9-desaturation of 12-18:1 has been postulated in essential fatty acid deficient rats (7), although no such desaturation could be found by other workers in the field (8,32). 6,12-18:2 is probably formed by Δ6-desaturation of 12-18:1. This monoenoic fatty acid could, however, not be detected in the present study although its occurrence in mammalian tissue is well documented (33-35). Similar pathways have to be postulated for 4,7-18:2 and 5,8-18:2 (36).

Concerning the structural requirements necessary for close interaction of the fatty acid substrates with the binding sites of the different desaturase systems, certain predictions can be made on the basis of the isomers listed in Table II. The biosynthesis of seven fatty acids 6,9-16:2; 6,9-18:2; 6,12-18:2; 6,9,12-18:3; 6-16:1; 6-17:1; 6-18:1 can be attributed to the action of a Δ6-desaturase system. General denominators for the substrates are chain lengths not exceeding 18 C atoms and double bonds, if any, in the same distances from the carboxyl group as in the genuine substrate 9,12-18:2. Eight different fatty acids were identified with double bonds in position 5: 5,8-18:2; 5,11-18:2; 5,11-20:2; 5,8,11-18:3; 5,8,11-19:3; 5,8,11-20:3; 5,11,14-20:3; and 5,8,11,14-20:4. They all posess at least one double bond in the same position as the genuine substrate of the Δ5-desaturase system at C-atom 8,11, or 14 with chain length between 18 and 20 C-atoms. A Δ4-desaturase system is postulated for the conversion of 7,10,13,16-22:4 into docosapentaenoic acid: 4,7,10,13,16-22:5 (37). The identification of 4,7,10,13-20:4 belonging to the (n-7) family shows that in fat deficient rats 7,10,13-20:3 is also accepted as a substrate.

These data indicate that the model for Δ6- and Δ5-olefinase originally proposed by Brenner may be extended and also specified for the Δ4-desaturase system (6,12). The structural requirements for acceptance as substrate can be defined generally by the following criteria: (a) The chain length must not exceed a minimal or maximal value, i.e. $C_{16/18}$ for the Δ6-; $C_{18/20}$ for the Δ5- and $C_{18/22}$ for the Δ4-desaturase system. (b) Already existing double bonds must be in the correct distance from the carboxyl group regardless of chain length, i.e. in position 9 for the Δ6-; in position 8,11 or both for Δ5- and in position 7,10,13 for the Δ4-desaturase system.

The analysis of unsaturated fatty acids as poly-OTMS derivatives by combined GLC-MS offers many advantages especially in the determination and identification of minor constituents of complex mixtures. The clearcut fragmentation of these derivatives under low energy electron impact and the high intensity of fragments of decisive diagnostic value allow the unequivocal identification of components poorly separated from major constituents as

well as of components occuring only in trace amounts. The extension of this method for the analysis of derivatives of penta and hexaenoic acids and the quantitation of the derivatives should widen our scope for the study of processes regulating the biosynthesis of polyenoic fatty acids.

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[Received November 15, 1976]

LETTER TO THE EDITOR

Preparation of Phosphorylcholine from Phosphorylcholine Chloride Calcium Salt

Sir: Phosphorylcholine is found in human and animal tissues and body fluids. It is a substantial constituent of the nervous tissue and plays an important role in phospholipid biosynthesis (1-3). It is also used as an intermediate for the synthesis of organic compounds having a phosphorylcholine moiety (4). Phosphorylcholine has been prepared from choline chloride and phosphoric acid (5-6), from diphenylphosphorylcholine chloride (7), and from phenylcholine phosphate (8).

These previously published methods are not well suited for the preparation of phosphorylcholine in gram quantities. The present letter describes the transformation of the commercially available calcium monochloride salt by a simple technique and in good yield. The method can be scaled up easily; it can also be used for the preparation of radioactively labeled compound.

The exchange of the chloride for hydroxide was effected by silver carbonate, and the calcium ions were replaced through the use of a weakly acidic methacrylic acid polymer resin with carboxylic groups. Stronger acidic cation exchangers would react with the anionic moiety of the liberated compound. Phophorylcholine was obtained in low yield by treatment of the calcium monochloride salt with a mixture of weakly acid and basic ion exchange resins (9). The present method also describes a new thin layer chromatographic system using Silica Gel G plates.

Procedure: Phosphorylcholine chloride calcium salt–4 H_2O, 66 g (0.2 mol), (Sigma Chemical Co., St. Louis, MO) was dissolved in 1 liter of water. To this solution was added 41.4 g (0.15 mol) silver carbonate [Fisher Scientific Co., Pittsburgh, PA, or it was prepared according to (10)] and the mixture was stirred for 16 hr in the dark. A filtered aliquot showed 0.12 mol of calcium ions, 6 mmol of residual silver ions and the absence of chloride ions (determined by methods published, ref. 11).

The mixture was passed through a 200 g Rexyn 102 H, (a methacrylic acid polymer with carboxylic group from Fisher Scientific Co.) 100-200 mesh column (4 x 25 cm) which was then washed with 400 ml of water. Tests for calcium ions in the effluent were negative.

The pooled solutions of phosphorylcholine were evaporated at 40 C (16 mm Hg) with a rotary evaporator and the residue was then further dried at the same temperature at 0.1 mm Hg for 4 hr. It weighed 35 g (87%), and was recrystallized from 200 ml of ethanol yielding 21 g; mp 231-232 C (decomp). Additional amounts of this compound could be obtained by reducing the volume of the mother liquor. A second recrystallization from 15 times (v/w) methanol-acetone (2:3) gave a product melting at 237-238 C (decomp.) (Taken on a Thomas-Hoover melting point apparatus and corrected). [Lit. (8); 234-238 C (decomp.)]. Phosphoryl-

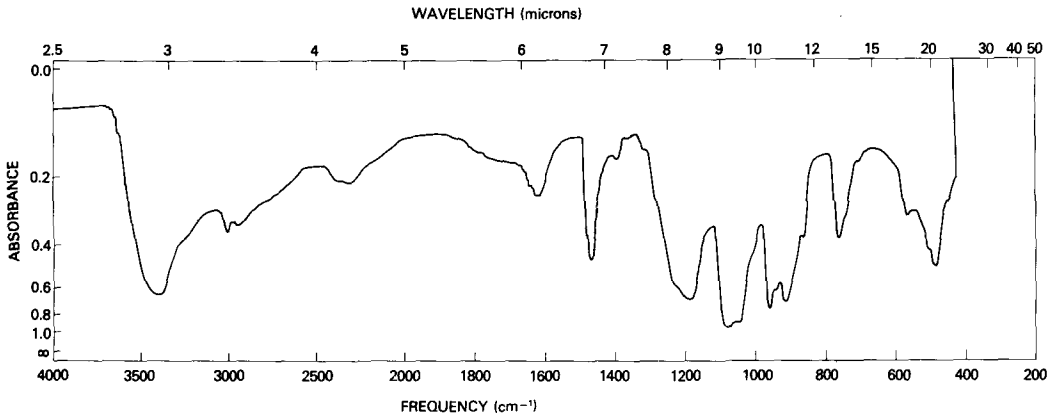

FIG. 1. The infared spectrum of phosphoryl choline.

choline is soluble in methanol (0.8 g/ml) and insoluble in acetone. Thin layer chromatography: on Silica Gel G plates in acetic acid–n-propanol–water (1:3:4). R_f 0.35, [visualized by using the phospholid spray solution of Vaskovsky and Kostetsky (12)]. The infared spectrum (Fig. 1) displayed bands at 3400 (hydroxy), 1460 (CH_3, CH_2), 1220-1180 (shoulder, P=O), 1080 (PO^-), 1040 (P-O-C), 960 and 910 (P-OH), and 760 cm^{-1} (-CH-). (Obtained with a Perkin-Elmer 621 infared grating spectrophotometer as a KBr disk, 1.5 mg sample/300 mg KBr). Anal. Calcd. for $C_5H_{16}NO_5P$ (201.16): C, 29.86; H, 8.02; N, 6.96; P 15.39. Found: C, 30.08; H, 7.90; N, 7.21; P, 15.35.

[Me-^{14}C] Phosphorylcholine. [Me-^{14}C] Choline chloride, (New England Nuclear, Boston, MA) 69.8 mg (0.5 mmol), 0.1 mCi, was heated at 70 C for 3 hr with a mixture of 85% phosphoric acid 250 mg and phosphorus pentoxide 175 mg. The mixture was cooled to 0 C, 0.35 ml of water was added and the excess acid was extracted twice with 3.5 ml of acetone. The remaining oil was dissolved in 0.5 ml of 1*N* hydrochloric acid and heated for 10 min at 100 C. After addition of 3 ml of water, the pH was adjusted to 8.2 with calcium hydroxide. The mixture was filtered and the filtrate evaporated to 0.5 ml. Addition of 0.5 ml of ethanol precipated 140 mg of the calcium salt of [Me-^{14}C] phosphorylcholine chloride. This salt was then transformed into [Me-^{14}C] phosphorylcholine by the above described procedure. Yield: 68 mg (68%), 0.2 mCi/mmol. Mp. 232-235 C (decomp.) (The radioactivity was measured with Packard Tricarb Model 3003, using Aquasol LSC cocktail from New England Nuclear).

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[Received August 25, 1976]

SHORT COMMUNICATIONS

Differential Biosynthesis of Molecular Species of 1,2-diacyl-*sn*-glycerols and Phosphatidylcholines in Cold and Warm Acclimated Goldfish (*Carassius auratus* L.)

ABSTRACT

The initial incorporation of glycerol-^{3}H into the molecular species of liver 1,2-diacyl-*sn*-glycerols and phosphatidylcholines was studied in vivo using goldfish acclimated to 10 C and 30 C. A 1.5- and 2.2-fold higher proportion of the total radioactivity in the diacylglycerols from cold acclimated fish was found to be associated with the trienoic and pentaenoic species, respectively, when compared to warm acclimated fish. In the phosphatidylcholines, 1.9- and 1.3-fold greater percentages of the newly-incorporated radioactivity were found in tetraenoic and pentaenoic molecules, respectively, from cold relative to warm acclimated fish which suggests a preferential synthesis of these molecules relative to other molecular species in response to a lowering of environmental temperature. The present results indicate, therefore, that environmental temperature influences the complement of molecular species of diacylglycerols and phosphatidylcholines which fish produce by way of de novo biosynthesis in vivo.

INTRODUCTION

There have been numerous reports in the literature to support the concept that the fatty acid composition of cellular phospholipids from fish and other ectothermic (poikilothermic) organisms is dependent upon the environmental temperature (1-4). It has been suggested that the increase in the degree of unsaturation of cellular lipid which is observed with a decrease in temperature reflects an attempt of such organisms to regulate membrane fluidity (5). Thus, there is considerable interest in the biochemical mechanisms used by aquatic ectotherms to control the types of fatty acyl moieties present in cellular phospholipid when the temperature of the ambient medium is altered. It has been suggested that the cytidine diphosphate (CDP)-choline:1,2-diacyl-*sn*-glycerol cholinephosphotransferase in fish (6) may produce a different complement of molecular species of lecithin at different environmental temperatures. Recent experiments in vitro have provided some support for this possibility with the demonstration that the proportion of newly-synthesized lecithins which were polyenoic species decreased as the incubation temperature was increased (7). The significance

TABLE I

Percent Distribution of Glycerol-^{3}H among Molecular Species of 1,2-diacyl-*sn*-glycerols in Goldfish Liver[a]

Molecular species	Warm acclimated	Cold acclimated	Cold/warm ratio
Saturates + monoenes	61.9 ± 2.1	53.0 ± 4.4	0.86
Dienes	15.2 ± 1.4	15.5 ± 5.3	1.02
Trienes	2.8 ± 0.3	4.3 ± 0.6*	1.54
Tetraenes	3.2 ± 0.6	3.9 ± 0.6	1.22
Pentaenes	2.0 ± 0.3	4.4 ± 0.5*	2.20
Hexaenes	14.9 ± 1.2	18.9 ± 3.1	1.27

[a]Values are given as % distribution of radioactivity among the various molecular species of diacylglycerols and are presented as means ± S.E. for separate analyses on five individual fish in each group. Fish from each group were sacrificed 5-15 min (one at 5 min, three at 10 min, and one at 15 min) after administration of the labeled glycerol. Small amounts of radioactivity remaining at the origin of thin layer chromatograms have been omitted from the calculated distributions. Lipid extracts contained 6-7% of the total radioactivity in livers of warm acclimated fish and 2-5% of that in cold acclimated fish. Total lipid extracts contained (5-20) x 10^4 cpm per liver. Values in cold acclimated group followed by an asterisk are significantly different from corresponding values in warm acclimated group ($P<0.05$).

TABLE II

Percent Distribution of Glycerol-^{3}H among Molecular Species of Phosphatidylcholines in Goldfish Liver[a]

Molecular species	Warm acclimated	Cold acclimated	Cold/warm ratio
Saturates	1.4 ± 0.8	1.1 ± 0.6	0.79
Monoenes	7.4 ± 0.6	9.0 ± 0.5	1.22
Dienes	4.5 ± 0.3	4.7 ± 0.6	1.04
Trienes	2.8 ± 0.2	3.5 ± 0.6	1.25
Tetraenes	2.5 ± 0.2	4.7 ± 0.9*	1.88
Pentaenes	7.3 ± 0.6	9.8 ± 0.5*	1.34
Hexaenes	74.1 ± 1.4	67.2 ± 2.1*	0.91

[a]See legend to Table I.

in vivo of these latter findings is unknown because of various limitations in interpreting such in vitro studies. The purpose of the present investigations was to compare the molecular species of hepatic 1,2-diacyl-*sn*-glycerols and the corresponding lecithins which are generated by de novo synthesis under physiological conditions in cold and warm acclimated goldfish.

EXPERIMENTAL PROCEDURES

Goldfish (*Carassius auratus* L.) were purchased from Hartz Mountain Pet Supplies and were maintained under a 12 hr photoperiod at 10±1 C or 30±1 C for a period of at least 9 wk. They were fed 1 to 3 times a day ad libitum with a commercial pelleted fish diet (Martin Feed Mills, Elmira, Ont.). Glycerol-2-^{3}H (192 mCi/mmole) was obtained from the New England Nuclear Corp., Boston, MA. For in vivo experiments, 100 μl of a saline solution containing labeled glycerol (100 μCi) was administered intraperitoneally. Livers were rapidly excised at appropriate time intervals and lipid extracts immediately prepared (8). Phosphatidylcholines and free 1,2-diacyl-*sn*-glycerols were isolated from total lipid extracts by elution from the gel scrapings following thin-layer chromatography (TLC) (9). Carrier lipid from rat liver was added where required.

The phosphatidylcholines were converted to their corresponding diacylglycerols using phospholipase C from *Clostridium welchii* (9). The distribution of radioactivity among the various molecular species of diacylglycerols was determined by scintillation counting (6) after separation of these fractions by argentation TLC and elution from the gel scrapings (10). Data were analyzed by one way analysis of variance after conducting an arcsin x $\sqrt{\text{percentage}}$ transformation (11).

RESULTS AND DISCUSSION

Table I gives the percent distribution of radioactive glycerol among the various molecular species of free diacylglycerols at 5-15 min after isotope administration. These particular times were chosen based on preliminary experiments which indicated that the amount of radioactivity appearing in the glycerolipid products was increasing dramatically and high levels of radioactivity were associated with the intermediary diacylglycerols in contrast to later times. The total cpm in hepatic lipids was greater at times later than 15 min (from 15-45 min) and the percent of total liver radioactivity in the lipid phase steadily increased relative to that in the aqueous phase at these later times. Glycerol-2-^{3}H was employed as a precursor in these experiments because its initial entry into glycerolipids via known metabolic pathways represents de novo synthesis of lipid (9,12,13). Although the isotopic distributions among the various molecular species of diacylglycerols were very similar at 5 to 15 min within each experimental group, striking differences were found between warm and cold acclimated fish. Thus, much higher proportions (1.5- and 2.2-fold) of the total radioactivity associated with the newly-synthesized diacylglycerols were found in the trienoic and pentaenoic species of cold acclimated fish as compared to warm. These latter differences were statistically significant ($P<0.05$). In the case of the phosphatidylcholines (Table II), the tetraenes and pentaenes contained a significantly higher proportion of the total radioactivity in cold as compared to warm acclimated fish whereas the opposite was true for the hexaenoic species. The differences in radioactivity distributions among the various molecular species of phosphatidylcholines as compared to the corresponding 1,2-diacyl-*sn*-glycerols (Table I) suggest a highly preferential utilization of pentaenoic and hexaenoic molecules of intermediary diacylglycerols for the de novo synthesis of lecithin at both acclimation temperatures.

In summary, the present results suggest that

the acclimation temperature of fish influences the complement of molecular species of precursor diacylglycerols synthesized de novo and of liver phosphatidylcholines which are subsequently formed in vivo by the CDP-choline: 1,2-diacyl-*sn*-glycerol cholinephosphotransferase. These findings indicate that the CDP-choline pathway produces a population of lecithins in cold acclimated fish which has a much higher abundance of tetraenoic and pentaenoic molecular species as compared to that produced in fish acclimated to higher temperatures. It remains to be established, however, to what extent these differences are controlled by enzyme selectivity and the availability of precursors of varying fatty acid compositions. The data in Tables I and II support the concept that both factors are of importance in regulating the complement of lecithins that are synthesized by the cholinephosphotransferase under physiological conditions. Finally, it is tempting to speculate that a change in the environmental temperature of a poikilothermic organism may elicit a corresponding change in membrane fluidity which mediates the biogenesis of appropriate molecular species of phospholipid in an attempt to maintain the fluidity of cellular membranes in harmony with normal cellular function.

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[Revision received December 22, 1976]

Sterol Composition and Phytosterol Utilization and Metabolism in the Milkweed Bug

ABSTRACT

Analysis of the sterols of the milkweed bug, *Oncopeltus fasciatus* (Dallas) and dietary sunflowerseeds revealed that there is little, if any, conversion of dietary C_{28} or C_{29} phytosterols to cholesterol in this phytophagous insect. The dietary sterols are apparently utilized with little alteration both during development to the adult stage and egg production, and cholesterol comprises <1% of the sterols in either adult males and females or in the eggs. The significance of these findings are discussed in light of the recent discovery that the C_{28}-ecdysone, makisterone A, is the predominant molting hormone in the enbryonated egg of the milkweed bug.

INTRODUCTION

Previously, phytophagous insects were generally believed to be capable of dealkylating and converting C_{28} and C_{29} phytosterols to the C_{27} sterol cholesterol, thus making this

the acclimation temperature of fish influences the complement of molecular species of precursor diacylglycerols synthesized de novo and of liver phosphatidylcholines which are subsequently formed in vivo by the CDP-choline: 1,2-diacyl-*sn*-glycerol cholinephosphotransferase. These findings indicate that the CDP-choline pathway produces a population of lecithins in cold acclimated fish which has a much higher abundance of tetraenoic and pentaenoic molecular species as compared to that produced in fish acclimated to higher temperatures. It remains to be established, however, to what extent these differences are controlled by enzyme selectivity and the availability of precursors of varying fatty acid compositions. The data in Tables I and II support the concept that both factors are of importance in regulating the complement of lecithins that are synthesized by the cholinephosphotransferase under physiological conditions. Finally, it is tempting to speculate that a change in the environmental temperature of a poikilothermic organism may elicit a corresponding change in membrane fluidity which mediates the biogenesis of appropriate molecular species of phospholipid in an attempt to maintain the fluidity of cellular membranes in harmony with normal cellular function.

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Analysis of the sterols of the milkweed bug, *Oncopeltus fasciatus* (Dallas) and dietary sunflowerseeds revealed that there is little, if any, conversion of dietary C_{28} or C_{29} phytosterols to cholesterol in this phytophagous insect. The dietary sterols are apparently utilized with little alteration both during development to the adult stage and egg production, and cholesterol comprises <1% of the sterols in either adult males and females or in the eggs. The significance of these findings are discussed in light of the recent discovery that the C_{28}-ecdysone, makisterone A, is the predominant molting hormone in the enbryonated egg of the milkweed bug.

INTRODUCTION

Previously, phytophagous insects were generally believed to be capable of dealkylating and converting C_{28} and C_{29} phytosterols to the C_{27} sterol cholesterol, thus making this

TABLE I

Milkweed Bug and Sunflowerseed Sterols Relative % Δ^5-Sterols

	♂ Adult		♀ Adult		Egg		
	Ester	Free	Ester	Free	Ester	Free	Sunflowerseed sterols
Cholesterol	0.4	0.5	0.3	0.4	0.9	0.5	<0.1
Campesterol	10.6	11.0	10.6	10.0	9.7	11.9	10.1
Stigmasterol	8.9	2.4	11.4	4.0	15.1	5.5	10.3
Sitosterol	80.1	86.1	77.7	85.6	74.3	82.1	79.5
% of Total Δ^5-Sterols	65.3	34.7	46.5	53.5	11.0	89.0	--
C_{30} Sterols as % of Total	9.8	4.4	10.3	5.8	10.8	4.1	19.1

sterol available for structural purposes and as a precursor for the C_{27} insect steroid molting hormones (ecdysones) (1). However, both the confused flour beetle, *Tribolium confusum* (Jacquelin du Val), and the Mexican bean beetle, *Epilachna varivestis* (Mulsant), were found to have unique sterol compositions that were shown to result from different pathways for the utilization and metabolism of C_{28} and C_{29} dietary sterols in these two plant-feeding insects (1-4). In addition, the recent isolation and identification of the C_{28} ecdysone makisterone A, the first C-24 alkyl substituted molting hormone isolated from an insect, as the predominant molting hormone of the 4-day old embryonated egg of the milkweed bug, *Oncopeltus fasciatus* (Dallas) (5), indicates the presence of heretofore unknown pathways for ecdysone biosynthesis in an insect. Apparently certain C_{28} or even C_{29} sterols, rather than first being converted to a C_{27} sterol such as cholesterol, may serve directly as precursors for molting hormone biosynthesis in this insect. The latter study prompted us to examine the sterol content and the utilization and metabolism of plant sterols in the milkweed bug to determine whether this plant-feeding insect is, in fact, unusual in its ability to utilize dietary plant sterols directly for ecdysone biosynthesis without first dealkylating these sterols to produce cholesterol or a related C_{27} sterol.

EXPERIMENTAL PROCEDURES

Three-week-old milkweed bug nymphs, reared on sunflowerseeds, were sexed and then maintained on the same diet for 2 more wk after molting to the adult stage. The insects were then held for 24 hr without food to reduce the food content of the gut and then weighed and stored frozen. Also eggs were collected from a laboratory colony reared on sunflowerseeds and frozen 4 days after collection. For neutral sterol analysis, adult females (15.2 g), adult males (9.8 g), and 4-day old eggs (5.2 g) were homogenized and extracted in chloroform-methanol (2:1). The crude lipid from this extract was fractionated by column chromatography on Unisil (Clarkson Chemical Co., Williamsport, PA) as previously described, to separate sterol esters from free sterols, and the column fractions were monitored by thin layer chromatography (TLC) (6). The sterol ester and free sterol fractions were saponified, and the recovered sterols were purified by column chromatography on alumina (3) and further purified by digitonide precipitation to remove accompanying fatty alcohols. The sterols were identified by gas chromatographic-mass spectral (GC-MS) analysis using an LKB model 9000 gas chromatograph mass spectrometer (LKB Produckter AB, Stockholm, Sweden). Samples were introduced into the ionization chamber (ionization energy 70 ev.) from a 0.75% SE-30 gas chromatography column. They were also identified and quantified by comparison of relative retention times (RRTs) of the insect sterols with those of authentic sterol standards by gas liquid chromatographic (GLC) analysis on a 1.0% OV-17 system in a Barber-Colman model 10 gas chromatograph equipped with a beta ionization detector cell.

Portions of the milkweed bug sterols were acetylated and chromatographed on 20% $AgNO_3$-impregnated Unisil as previously described (2). The column fractions were monitored on 20% $AgNO_3$-impregnated Silica Gel H chromatoplates developed in benzene-*n*-hexane (1:1) to determine whether this insect contained a significant level of saturated or Δ^7- and $\Delta^{5,7}$-unsaturated sterols, as had been found in the Mexican bean beetle (3) or the confused flour beetle (2). No significant amount of saturated or Δ^7- and $\Delta^{5,7}$-unsaturated sterols was found in the insect sterol samples.

Each of a group of early 5th-instar nymphs was injected with 0.1 μl of a 10% dimethyl

formamide suspension containing 0.033 μg [2,4-^{3}H]-sitosterol (sp. act. 24,000 cpm/μg) having a radiochemical purity >97%, as established by radiochromatogram scanning of TLC chromatoplates and trapping effluent fractions from GLC. Immediately after the adult molt, 24 insects were homogenized and the sterols extracted and isolated. These sterols contained 63% of the original injected radioactivity (11,900 cpm). Fractions of these sterols were trapped from the GLC effluent and counted on a Packard Tricarb Scintillation Spectrometer to determine whether ^{3}H-sitosterol was converted to cholesterol by this insect.

RESULTS AND DISCUSSION

The Δ^5-sterols listed in Table I were identified by GC-MS and by their RRTs in the OV-17 GLC system. There is little if any alteration of the dietary sterols by this insect, except for a notable increase in the relative percentage of cholesterol. Even though cholesterol is present in such a low concentration in sunflowerseed sterols (<0.1%), its presence was verified by GC-MS and very possibly selective absorption of this dietary sterol could account for all the cholesterol found in the milkweed bugs (ranging from 0.3 to 0.9% in various samples). Thus, this insect is quite likely incapable of dealkylating C_{28} and C_{29} phytosterols. Similar results were previously obtained from adult milkweed bugs fed milkweed seeds, using less purified sterol samples and tentative identifications based on absolute retention times on a single GLC system (7). However, in this case, no cholesterol could be detected in milkweed seeds, so it was concluded that both the unequivocal identification of the insect sterols and studies with radiolabeled phytosterols were needed to determine the amount of phytosterol conversion that occurs in the milkweed bug (7).

There is also an apparent sequestering of stigmasterol in the ester sterols of the eggs of the milkweed bug (Table I). In addition, an interesting difference in the ratios of esterified and free sterols occurred between males, females, and eggs, which suggests that free sterols may be most readily available for ovarian development in the female and for embryonic development in the egg. The adult male had only 34.7% of the total Δ^5-sterols present as free sterol; the adult female and egg had 53.5 and 89.0%, respectively, of the Δ^5-sterols available as free sterols. The values for the sterol esters in both sexes of adults are higher than those found for the Mexican bean beetle (4).

A significant quantity of C_{30} sterols (Table I) derived from the dietary sterols is transferred in the sterol pool through all stages of the insect, including the egg. They accompany the normal Δ^5-sterols through the digitonide precipitation, and analysis by GC-MS indicates that these sterols are similar to those that commonly occur in plant material.

All phytophagous insects we have worked with, including the Mexican bean beetle (4), that are able to dealkylate phytosterols are also capable of readily converting sitosterol to cholesterol or related C_{27} sterols. Upon examining the sterols from milkweed bugs injected with ^{3}H-sitosterol, we found no detectable conversion of this phytosterol to cholesterol by trapping fractions from the GLC effluent and determining radioactivity of the recovered material. No more than background activity could be found in the fractions corresponding to cholesterol when four aliquots containing 200-250 cpm were injected and trapped (~50% of injected radioactivity recovered). This further substantiates the premise that these insects are unable to dealkylate plant sterols to produce cholesterol.

The report that makisterone A, a C_{28} ecdysone, is the major molting hormone of milkweed bug eggs, and that 96- and 120-hr-old eggs of the milkweed bug contain seven to ten times more housefly units of ecdysone activity than 1-day-old eggs (5), indicates that the developing embryo of the milkweed bug has the capacity to produce its ecdysone from a C_{28} sterol or steroid precursor. The present findings suggest that the Δ^5-C_{28}-sterol campesterol could be a likely precursor of makisterone A and therefore support the proposal of Kaplanis et al. (5) that certain species of insects are able to utilize C_{28} plant sterols directly as precursors for their molting hormones. This occurs in the case of the milkweed bug embryonated egg even though there may be sufficient cholesterol available for ecdysone production, indicating that this insect can perhaps better utilize campesterol than cholesterol for molting hormone biosynthesis. It is therefore quite likely that this insect can develop on a diet completely devoid of cholesterol.

These studies with the milkweed bug, taken together with our previous results with the Mexican bean beetle and the confused flour beetle, indicate that a considerable diversity in sterol utilization and/or metabolism exists between different species of plant-feeding insects. Adaptations to the numerous ecological niches and food sources utilized by insects then must have quite often been accompanied by corresponding physiological and biochemical adaptations in steroid metabolism. This appears

to be true not only in the case of neutral sterol metabolism, but with ecdysone metabolism as well (5) and it will be of interest to determine the nature and the extent of the variations in steroid metabolism that have occurred in other insect species. It will also be particularly interesting to determine whether the ecdysones and the intermediates involved in the pathways for the C-24 alkyl substituted molting hormones in insects, such as the milkweed bug, are similar or homologous to those known for the C_{27} molting hormones of insects.

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[Received November 15, 1976]

Myristicin. The Major Volatile Component in Mature Seed of *Portenschlagia ramosissima*

ABSTRACT

Portenschlagia ramosissima (Port.) vis. (Umbelliferae) seed contains 15% essential oils, 70% of which is the aromatic ether myristicin. These structural assignments were made from nuclear magnetic resonance, combined gas chromatography-mass spectrometry (GC-MS), and infrared and ultraviolet data. GC-MS data also indicate the presence of pinene, cymene, terpinene, elemicin, methyl eugenol, and a variety of sesquiterpene hydrocarbons.

INTRODUCTION

Biological active phenylallyl derivatives have been reported as constituents in a variety of *Umbelliferae,* many of which are edible plants (1-3). Many of these derivatives, especially myristicin and elemicin, produce physiological effects in man (2) and exhibit pesticidal properties with some insect species (3). We now report that the volatile oil (15%) from the mature seed of *Portenschlagia ramosissima,* collected in Yugoslavia and sometimes referred to as *Portenschlagiella* (1), contains both myristicin (70%) and elemicin (1.5%). However, these phenylallyl derivatives were not reported in this species by other investigators (1,4). Since our sample of *P. ramosissima* is the richest source of myristicin known (greater than 10% of the total seed weight), we do not know whether the discrepancy between our work and that of others (1,4) is due to misidentification of the plant species, differences in genetic material within the species, agronomic conditions during production, or differences in chemical methodology.

EXPERIMENTAL PROCEDURES

A sample of the volatile oil was obtained from the ground seed by two methods: (A) by treatment in a steam distillation continuous extraction apparatus of the type described by Likens and Nickerson (5), and (B) by extraction according to the method described by Harborne and coworkers (1). The volatile oil obtained from (A) was fractionated by preparative thin layer chromatography (TLC) on plates coated with a 1-mm thick layer of Silica Gel G and developed with benzene. The extracted oil

to be true not only in the case of neutral sterol metabolism, but with ecdysone metabolism as well (5) and it will be of interest to determine the nature and the extent of the variations in steroid metabolism that have occurred in other insect species. It will also be particularly interesting to determine whether the ecdysones and the intermediates involved in the pathways for the C-24 alkyl substituted molting hormones in insects, such as the milkweed bug, are similar or homologous to those known for the C_{27} molting hormones of insects.

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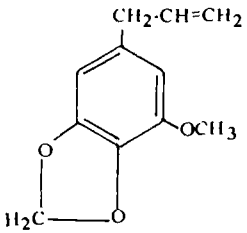
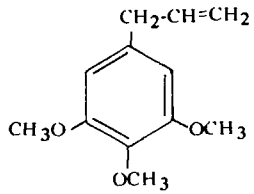
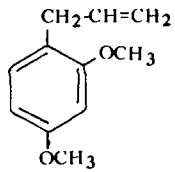
TABLE I

Mass Spectral and Nuclear Magnetic Resonance (NMR) Data for Three Toxic Components in *P. ramossisima*

Name, molecular weight and formulas	Structure	Mass spectrum[a] m/e (relative intensity)	NMR spectrum[b] δ (mult. and assign.)
Myristicin MW = 192 $C_{11}H_{12}O_3$		193 (21), 192 (M^+, 100), 191 (17), 165 (27), 161 (19), 147 (17), 133 (18), 131 (20), 119 (27), 91 (46), 77 (24) 65 (26) SI[c] = 0.9	3.30 (br. d., $-CH_2-$) 3.90 (s, $-OCH_3$) 5.10 (br. d., $CH_2=$) 5.93 (br. m., CH=) 5.94 (s, $-OCH_2O-$) 6.40 (br. m., Aromatic H)
Elemicin MW = 208 $C_{12}H_{16}O_3$		209 (26), 208 (M^+, 100), 194 (12) 193 (62), 177 (14), 165 (13), 150 (12), 135 (10), 134 (10) 133 (19), 118 (15), 105 (13), 91 (16), 79 (21), 78 (12), 77 (25), 65 (11) SI = 0.8	3.30 (br. d., $-CH_2-$) 3.90 (s, $-OCH_3$) 5.10 (br. d., $CH_2=$) 5.93 (br. m., CH=) 6.38 (s, Aromatic H)
Methyl eugenol MW = 178 $C_{11}H_{14}O_2$		179 (20), 178 (M^+, 100), 163 (39) 151 (15), 147 (36), 135 (16), 115 (12), 107 (31), 105 (15), 103 (36), 92 (11), 91 (44), 79 (20), 78 (12), 77 (22), 65 (16), 51 (12), 41 (18), 39 (17) SI = 0.8	3.30 (br. m., $-CH_2-$) 3.82 (s, $-OCH_3$) 5.10 (br. d., $CH_2=$) 5.93 (br. m., CH=) 6.70 (br. m., Aromatic H)

[a]Only peaks >10% of base listed.

[b]Data in parentheses: multiplicity (s, singlet; d, doublet; m, multiplet; br., broad) and assignment.

[c]Similarity index.

from (B) was separated in a similar manner except that a mixture (80:20 v/v) of *n*-hexane and diethyl ether was used for development. After spraying with a 0.2% alcoholic solution of 2,4-dichlorofluorescein and exposure to ultraviolet (UV) light, four bands (Fractions I-IV) from the steam volatile oils and two major bands (Fractions V-VI) from the extracted oils were visible. Each band (Fractions I-VI) was scraped from the plates and the components were eluted from the adsorbent with Et_2O. The samples were then concentrated under nitrogen at room temperature and analyzed by gas liquid chromatography (GLC) in a 4 ft x 1/4 in. glass column packed with 3% Apiezon L and a 6 ft x 1/4 in. glass column packed with 5% LAC-2-R 446. Tentative identification of the volatile oils is based on comparisons of GLC retention characteristics with known compounds. Percentages of individual components are relative area percentages, uncorrected for detector response. The columns for the volatile oils were temperature-programmed from 70-200 C at 4 C/min. GLC data of the triglycerides (Fraction VI) were obtained by the method described by Litchfield et al. (6). Infrared, UV, nuclear magnetic resonance (NMR), and mass spectrometry (MS) data were obtained as previously described (7), except a Bendix Model 2625 GC was used in tandem with the MS. Methyl eugenol was prepared by methylation of eugenol with CH_2N_2.

RESULTS AND DISCUSSION

The steam volatile oil (5) was separated by TLC into four classes of compounds [fraction I (R_f = 0.99), fraction II (R_f = 0.70), fraction III (R_f = 0.27) and fraction IV (R_f = 0.10)]. GC-MS data of the individual fractions indicate that fraction I (26.7% of the volatile oil) is a mixture of α-pinene 0.8%, β-pinene 1.7%, cymene 42%, γ-terpinene 50%, and trace amounts (<0.1% each) of several sesquiterpene hydrocarbons. Mass spectral data of the individual components and those of authentic samples were in agreement with reference spectra when compared by means of a computerized method described by Hertz et al. (8). Such comparison can be expressed in a similarity index (SI). SI = 0 denotes no similarity in fragmentation patterns and SI = 1 indicates complete agreement between test material and reference compound. Any value of SI above 0.5 is considered a good match.

The major component, 70% of the volatile oil and 98% of fraction II, was identified as the aromatic ether myristicin. Pertinent MS and NMR data supporting the structure of myristicin are shown in Table I. The MS and NMR data are consistent with data obtained from a commercial sample of myristicin and those reported in the literature (3).

GLC retention time of the major component (82%) in fraction III (1.4% of volatile oil) was identical to that of known methyl eugenol. NMR and MS data of the isolated methyl eugenol (Table I) confirmed its structure. Also present in fraction III, indicated only by comparison with reference spectra are: thymol (4.0%), thujyl alcohol (0.3%), and a suspected sesquiterpene alcohol (5.0%). The major component (87%) in fraction IV (1.6% of the volatile oil) was identified as elemicin. This compound was identified by its MS fragmentation pattern and NMR data as shown in Table I.

Although not analyzed as detailed as the steam volatile oil, composition of the extracted oil (1) was investigated. The extracted oil contains a large amount of triglycerides indicated by TLC (R_f = 0.45) in addition to the volatile oil. Compositions of the volatile oils obtained by either extraction or steam distillation methods (1,5) are identical as shown from GLC data. NMR and GLC data confirmed the structure of triglycerides for the component(s) at R_f 0.45 (6,7).

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A Simple Method for the Preparation of Pure 9-D-Hydroperoxide of Linoleic Acid and Methyl Linoleate Based on the Positional Specificity of Lipoxygenase in Tomato Fruit

ABSTRACT

Incubation of linoleic acid with crude homogenate of tomato fruit gave a high yield (69%) of linoleic acid hydroperoxides with a ratio of 9- to 13-hydroperoxide isomers of 96:4. After chromatography of the products, as free acids or methyl esters, hydroperoxides with 9- to 13-isomeric ratios of >99:1 were obtained. The major product was characterized as 9-D-hydroperoxy-octadeca-*trans*-10,*cis*-12-dienoic acid. The results demonstrate the positional specificity of lipoxygenase from tomato fruit.

INTRODUCTION

Fatty acid hydroperoxides are important intermediates in fatty acid metabolism and in lipid oxidation processes. Our studies on enzymic degradation of fatty acids and on non-enzymic lipid oxidation demanded separate preparations of pure 9- and 13-hydroperoxides of linoleic and linolenic acids.

13-L-Hydroperoxy-octadeca-*cis*-9,*trans*-11-dienoic acid can now be produced in better than 99% purity using commercial preparations of soybean lipoxygenase at high pH followed by chromatographic purification (1-4). The 9-hydroperoxide is less readily produced; but reasonably pure (95-97% of 9-isomer) preparations in moderate yield are obtained using lipoxygenases from potato (5) or corn germ (2). The present work is based on the high specificity of tomato lipoxygenase for 9-hydroperoxides and the presence of a 13-hydroperoxide-specific cleavage system in tomato fruits (6). We have been able to develop a simple method of producing high yeilds of very pure 9-D-hydroperoxides.

MATERIALS AND METHODS

Firm, red tomato fruits were peeled and, after seeds were removed, the flesh was diced and washed in H_2O at 0 C. Linoleic acid (Sigma Chemical Co., London) and [1-^{14}C] linoleic acid (Radiochemical Centre, Amersham) were mixed (sp. radioactivity: 0.03 μCi/μmole) and converted to the ammonium salt. To the diced tomato tissue (225 g) were added 750 ml of 0.1 M NaOAc buffer (pH 5.5) and ammonium linoleate (1 mM final concentration). The mixture was then homogenized (Ultra-Turrax) and incubated 15-20 min at 25 C in an open vessel. Oxygen was passed through the mixture for ca. 15 sec every 2 min. The products were extracted with chloroform and the extract was dried over Na_2SO_4. Different preparations using from 0.4 mmole to 1 mmole of linoleic acid gave similar conversion efficiencies. Analysis of the products from [^{14}C]-labelled linoleic acid, as determined by radioscanning (5), gave the following approximate thin layer chromatography (TLC) product distribution: fatty acid hydroperoxide, 70%; unreacted fatty acid, 16%; other minor products, 14%. The solvent was removed in vacuo. Subsequent treatment depended upon whether free acids or methyl esters were produced.

Free acid products were dissolved in light petroleum (b.p. 60-80 C)-diethyl ether 97.5:2.5 v/v and were separated on a column (26 cm x 1.5 cm ID) of EDTA-washed silicic acid as previously described (7). Elution was achieved by a gradient of diethyl ether in light petroleum (b.p. 60-80 C) with a mixing volume of 150 ml followed by 80 ml diethyl ether. Fractions (10 ml) were analyzed for ^{14}C-counts, and A_{234}-nm (in ethanol) components were identified by TLC (7) and, after methylation, by high performance liquid chromatography (HPLC) (3) which also determined the ratio of 9 to 13 isomers present in the fractions.

For the preparation of pure methyl hydroperoxydienoates, the chloroform-soluble incubation products were treated with CH_2N_2 and the esters were separated on a column (15 cm x 1.5 cm) of "Hiflosil" (Applied Science Laboratories, Inc, State College, PA) (8).

The column was eluted with light petroleum (b.p. 67-70 C)-diethyl ether mixtures 90:10 v/v (100 ml) followed by 80:20 v/v (100 ml). Fractions (8 ml) were collected and those giving a positive peroxide spot test (8) were analyzed by A_{234nm} (in ethanol) and by HPLC (3).

RESULTS AND DISCUSSION

Free 9-hydroperoxyoctadecadienoic Acid Preparation

When the products formed from 140 mg (13.5 μCi) of [1-^{14}C]linoleic acid were separated on a silicic acid column, early fractions from the gradient elution (30-120 ml) con-

tained unreacted linoleic acid (16% of total radioactivity) and traces (<1%) of a component, tentatively identified as a conjugated ketodienoic acid. Later fractions of the gradient (120-150 ml) and early fractions of the diethyl ether eluate (0-40 ml) contained only hydroperoxydienoic acid (69%). Subsequent fractions contained traces (1%) of hydroperoxide mixed with hydroxydienoic acid (5%) followed by unidentified polar products (9%). When uncontaminated fractions were combined, 99.7 mg of pure hydroperoxydienoic acid were obtained with a 9-:13-isomeric ratio of 96:4. However, as expected (2), fractions on the front of the peak contained relatively higher proportions of the 13-hydroperoxide isomer. Successive 10 ml fractions across the peak gave the following 9-:13-isomeric ratios and yields of hydroperoxide: 78:22 (6 mg), 95:5 (33 mg), 98:2 (22.2 mg), 99:1 (22.0 mg), >99:1 (9.0, 3.8, and 1.5 mg). Hydroperoxydienoic acid with a very high (>99:1) proportion of the 9-isomer was obtained at the expense of reduced yield (36.3 mg) by combining only those fractions with 9-:13- isomeric ratios ≥99:1.

Methyl 9-hydroperoxyoctadecadienoate Preparation

The methylated products from 50 mg linoleic acid gave an analogous isomeric ratio distribution across the hydroperoxide peak eluted from a silicic acid column. Successive 8 ml fractions gave the following 9-:13- isomeric ratios and yields: 60:40 (0.4 mg), 84:16 (2.1 mg), 94:6 (2.8 mg), 97:3 (5.5 mg), 98:2 (4.7 mg), 99:1 (2.6 mg), and >99:1 (1.4 mg). Combination of fractions with isomeric ratios ≥ 94:6 gave 17 mg (30% yield) of methyl hydroperoxydienoate containing 98% of the 9-hydroperoxy isomer.

Identification of the Hydroperoxydienoic Acid Product

The major product cochromatographed in HPLC with an authentic sample of the methyl ester of '9-D-hydroperoxide' (5). The 9-D-hydroperoxyoctadeca-*trans*-10,*cis*-12-dienoic acid obtained gave the following analyses as methyl ester. The molar ratio of peroxide (9) to conjugated diene (ϵ_{234} = 26,000) was 1:1.03; the infrared spectrum (CCL_4) had absorption bands at 951 and 989 cm^{-1} (*cis, trans*-conjugated diene); and the nuclear magnetic resonance spectrum was essentially identical with that of methyl 13-hydroperoxyoctadeca-*cis*-9-*trans*-11-dienoate (10) (*trans* double bond adjacent to the hydroperoxide group). The optical rotation of the hydroxy derivative obtained by $NaBH_4$ reduction gave $[\alpha]_D^{25}$ = +4.7° (3.1% in hexane), similar to published values (11-13). The methyl hydroxystearate, prepared and analyzed as previously (3), gave major fragment ions at m/e values of 155, 158, and 187 (characteristic of methyl 9-hydroxystearate). The corresponding characteristic ions of the 13- hydroxy isomer at m/e = 211, 214 and 243 were present in only minor amounts (<5% of the analogous 9-isomer fragments), confirming the HPLC data on isomeric distribution.

This work demonstrated that good yields of high purity 9-D-hydroperoxide of linoleic acid can be obtained from readily available materials and without prior enzyme purifications. The high proportion of 9-hydroperoxide isomer produced in this system is due to the previously unrecognized positional specificity of tomato lipoxygenase and to the presence in the tomato fruit of a hydroperoxide cleavage enzyme that is highly specific for the 13-hydroperoxide, thus selectively removing this isomer from the incubation system (6). A further advantage of the present method is that high yields of hydroperoxide product are obtained. It appears that the product inhibition usually experienced with lipoxygenases (14) is less marked in the crude tomato extracts.

Preliminary results with linolenic acid have shown that the method is also applicable to the preparation of the analogous 9-hydroperoxytrienoic acid. We do not know to what extent the small amount of 13-hydroperoxide in our preparations are due to residual products of enzyme activity or to autoxidation and isomerization (8) processes during the incubation and extraction procedures. Preparations of hydroperoxy acids and of their methyl esters are stable for several days in the dark at -20 C as dilute solutions in hexane-diethyl ether. A slow isomerization of 9- to 13-isomers occurs over a period of weeks. Isomerization is accelerated at higher concentration and temperature (8).

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Diet-induced Changes in the Fatty Acid Composition of Mouse Hepatocyte Plasma Membranes

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ABSTRACT

Hepatocyte plasma membranes were isolated from the livers of mice fed either a low fat diet or high fat diets containing polyunsaturated or saturated fat. The combined rate and isopycnic ultracentrifugation technique which was used produced highly purified hepatocyte plasma membrane fractions. The efficacy of the procedure was checked by electron microscopy and the assay of marker enzymes for the different subcellular organelles. Mice were maintained on a low fat diet until 60-70 days of age, when they were fed high fat diets containing polyunsaturated or saturated fat. The hepatocyte plasma membrane lipids of mice fed the polyunsaturated fat diet for 4 wk contained increased proportions of the major dietary unsaturated fatty acid, linoleic acid, and increased proportions of arachidonic acid. The proportion of linoleic and arachidonic acids decreased with continued feeding of the polyunsaturated fat diet. The hepatocyte plasma membrane lipids of mice fed the saturated fat diet contained increased proportions of oleic acid.

INTRODUCTION

It is now well established that the fatty acid composition of lipids from various tissues of monogastric animals reflects the fatty acid composition of dietary lipids (1-3). The fatty acid compositions of a number of subcellular organelles have been determined but there have been differences between the results, which may have been produced by different dietary conditions (4). This view is supported by the work of Witting et al. (5) who have shown that the fatty acid composition of rat liver mitochondrial lipids is readily altered by varying the fatty acid composition of the diet. Similarly, the fatty acid composition of rat erythrocyte ghosts reflects the unsaturated fatty acid composition of the dietary lipids (5-7). Although studies of dietary induced changes in fatty acid composition of such subcellular organelles have been reported, we are unaware of any such studies on plasma membranes.

Changes in the fatty acid composition of plasma membrane lipids are of physiological significance. Artificial and natural membranes containing unsaturated lipids have been shown to be more permeable than those containing more saturated lipids (8-13). Changes in the fatty acid composition of membranes also produce changes in the membrane lipid phase transition and breaks in the Arrhenius plots of the activities of lipid-associated membrane-bound enzymes (14).

It was, therefore, of interest to examine the changes induced by diet in the fatty acid composition of plasma membranes. In this preliminary study, we have determined the fatty acid composition of hepatocyte plasma membrane fractions isolated from the livers of mice transferred from a low fat diet to high fat diets containing either polyunsaturated or saturated fat.

MATERIALS AND METHODS

Animals and Diets

The C3HA^{vy}fB mice were bred in the Animal Breeding Establishment of the Australian National University from stock obtained from Dr. George Vlahakis at the National Cancer Institute, Bethesda, MD. At 60-70 days of age, the mice were transferred from a low fat diet, which they had been fed since birth, to high fat diets containing either saturated or polyunsaturated fat. The proportions of ingredients, proximate analyses and fatty acid compositions of the three diets are given in Tables I to III, respectively.

Mice were killed after being fed either of the high fat diets for zero, 2 or 4 wk. After 4 wk, the diets of the remaining mice were interchanged so that animals previously fed the saturated fat diet were fed the polyunsaturated fat diet and vice-versa. Mice were maintained for a further 2, 4, or 26 wk. In a second experiment, mice were killed after being fed either of the high fat diets for 2, 4, or 8 wk. The results

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TABLE I

Compositions of the Low Fat Diet and High Fat Diets Containing either Polyunsaturated or Saturated Fat

Ingredient	Low fat diet	High fat diets	
		Polyunsaturated	Saturated
Wheat	17.51[a]	41.4	41.4
Wheat bran	10.0	–	–
Wheat pollard	9.18	–	–
Barley	14.99	–	–
Oats	10.0	–	–
Maize	10.0	–	–
Dehydrated lucerne meal	–	5.5	5.5
Soyabean meal	4.17	32.0	32.0
Meat meal	13.9	–	–
Fish meal (65% protein)	10.0	–	–
Dicalcium phosphate	–	0.5	0.5
Lime	–	2.5	2.5
Vitamin and mineral premixes	0.25[b]	0.5[c]	0.5[c]
Methionine	–	0.1	0.1
Tallow	–	–	17.5
Sunflowerseed oil	–	17.5	–

[a]Values are % composition by weight.

[b]The vitamin and mineral premix supplemented the diet to the following amounts per kg total diet: vitamin A, 7,333 IU; vitamin D_3, 1,465 IU; vitamin E, 22 IU; vitamin B_1, 1.43 mg; vitamin B_2, 2.86 mg; vitamin B_6, 1.43 mg; vitamin B_{12}, 7.3 μg; vitamin C, 25 mg; nicotinic acid, 22 mg; pantothenic acid, 7.3 mg; iron, 50 mg; copper, 12.5 mg; cobalt, 1 mg; iodine, 1.5 mg; manganese, 75 mg; zinc, 55 mg; magnesium, 150 mg.

[c]The vitamin and mineral premix supplemented the diets to the following amounts per kg total diet: vitamin A, 13,200 IU; vitamin D_3, 1320 IU; vitamin E, 53 IU; vitamin K_3, 4.9 mg; vitamin B_1, 12 mg; vitamin B_2, 27 mg; vitamin B_6, 12 mg; vitamin B_{12}, 12 μg; vitamin C, 233 mg; biotin, 66 μg; folic acid, 7.3 mg; nicotinic acid, 146 mg; pantothenic acid, 43 mg; p-aminobenzoic acid, 49 mg; inositol, 244 mg; choline, 480 mg; iron, 70 mg; copper, 20 mg; cobalt, 50 μg; iodine, 1.6 mg; manganese, 60 mg; zinc, 60 mg.

of other experiments in this laboratory have shown that there is no difference in the growth rate of mice fed either of the high fat diets.

Isolation of Plasma Membranes by Zonal Centrifugation

Mice were killed by decapitation and their livers perfused *in situ* through the portal vein with Ca^{2+}-free Hank's solution containing 38mM-sodium citrate (15). A pooled sample of 4 g of liver from two or three mice was forced through a stainless mesh (1.18 mm aperture) into 36 ml of 0.25M-sucrose. All sucrose solutions used were adjusted to pH 7.6 with sodium bicarbonate. The tissue suspension was forced through the mesh and the fibrovascular skeleton retained on the mesh discarded. The tissue suspension was homogenized by hand using a Potter-Elvehjem homogenizer and was then filtered twice through a double layer of nylon cloth (0.18 mm aperture) and centrifuged at 400 x g for 10 min. The resulting nuclear pellet was resuspended in 36 ml of homogenizing medium with three strokes of a Potter-Elvehjem homogenizer (clearance 0.38 mm) with the teflon pestle rotating at 1,000 rev/min. All procedures were carried out at 4 C. Hepatocyte plasma membranes were isolated using the zonal centrifugation method of Gavard et al. (16) involving a combined rate and isopycnic banding separation. Samples for enzyme analysis either from the zonal separation or

TABLE II

Proximate Analyses of the Low Fat Diet and High Fat Diets Containing Either Polyunsaturated or Saturated Fat

Component	Low fat diet	High fat diets
Protein	22.4[a]	20.5
Fat	4.5	18.6
Fibre	5.1	4.1
Calcium	2.15	1.16
Phosphorus (available)	1.17	0.52
Phosphorus (total)	1.45	0.76
Methionine	0.43	0.40
Methionine and cysteine	0.84	0.80
Lysine (available)	1.11	1.11
Lysine (total)	1.31	–[b]
Leucine	1.38	–
Isoleucine	0.8	–
Tryptophan	0.26	–
Choline	–	0.18

[a]Values are % composition by weight.

[b]Not estimated

TABLE III

Fatty Acid Compositions of the Low Fat Diet and High Fat Diets Containing Either Polyunsaturated or Saturated Fat

Fatty acids	Low fat diet	Polyunsaturated fat diet	Saturated fat diet
14:0[a]	1.3 ± 0.07[b]	Trace[c]	3.2 ± 0.05
16:0	20.1 ± 0.26	9.1 ± 0.03	28.0 ± 0.05
16:1	–	Trace[c]	2.3 ± 0.05
18:0	7.4 ± 0.08	3.5 ± 0.10	19.3 ± 0.05
18:1	31.0 ± 0.31	27.2 ± 0.20	37.1 ± 0.15
18:2	36.0 ± 0.26	59.0 ± 0.05	10.1 ± 0.05
18:3	4.2 ± 0.17	1.2 ± 0.05	–

[a]Number C atoms: number double bonds.

[b]Values are mean ± standard error of 3 determinations on each of two samples of feed. Units are mole % of total fatty acids.

[c]<0.5%.

from liver homogenates (10% fresh weight by volume) were stored overnight at 4 C. Samples to be analyzed for fatty acid composition of lipids were dialyzed against distilled water overnight at 4 C, lyophilized, and stored at -15 C.

Enzyme Assays

The activities of the following enzymes were used as markers of various subcellular organelles: 5′-nucleotidase (EC 3.1.3.5) for plasma membranes; glucose-6-phosphatase (EC 3.1.3.9) for endoplasmic reticulum; acid phosphatase (EC 3.1.3.2) for lysosomes; and succinate dehydrogenase (EC 1.3.99.1) for mitochondria. The assays of the marker enzymes except succinate dehydrogenase were based on 30 min incubations at 37 C with an appropriate substrate. The assays of 5′-nucleotidase and glucose-6-phosphatase involved the release of inorganic phosphate from sodium adenosine monophosphate (AMP) (17) and glucose-6-phosphate disodium salt (18) respectively. Potassium sodium(+)tartrate was added to the 5′-nucleotidase assay medium as an inhibitor of acid phosphatase (19). Acid phosphatase was assayed by measuring the production of p-nitrophenol from p-nitrophenolphosphate (20) in citrate buffer at pH 5. Succinate dehydrogenase was assayed by measuring the rate of reduction of 2,6-dichlorophenolindophenol in the presence of sodium succinate (21).

As sucrose interferes with the assay of several enzymes, the results of the 5′-nucleotidase assay were corrected for sucrose interference (22). This was necessary because assays were performed directly on zonal fractions. Such a correction was not necessary in the assays of the other marker enzymes as the interference from sucrose was negligible due to the large dilution of zonal fractions in the incubation media.

Chemical Analysis

The inorganic phosphate released in the assays of 5′-nucleotidase and glucose-6-phosphatase was estimated as described by Eibl and Lands (23). The sucrose interference with this assay was quantitated and the results were corrected. The concentration of protein in various zonal fractions was estimated from the absorbances at 280 and 260 nm.

Electron Microscopy

Electron microscopy was also used to identify and characterize the plasma membrane fraction. Samples of fractions containing plasma membranes were smeared on collodion-coated copper grids, negatively stained with phosphotungstic acid (1% w/v) neutralized with 0.1N-NaOH and examined with an electron microscope.

Analysis of Fatty Acids

Lipids were extracted from aqueous suspensions of plasma membranes with chloroform-methanol (2:1, v/v) (24) to which butylated hydroxytoluene (0.005% w/v) had been added to prevent lipid autoxidation (25). Fatty acids were transesterified using 2,2-dimethoxypropane (26) and the fatty acid methyl esters analyzed by gas liquid chromatography. In the first experiment, samples were analyzed using two stainless steel columns. One column was packed with 10% EGSS-X on 80/100 Gas-Chrom Q and the other with 3% JXR on 100/120 Gas-Chrom Q (Applied Science Laboratories Inc., State College, PA). The fatty acid methyl esters were identified by comparing relative retention times and carbon numbers, calculated using a computer (27) with those of standards obtained from Nu-Chek-Prep Inc., Elysian, MN. Hydrogenation of the unsaturated

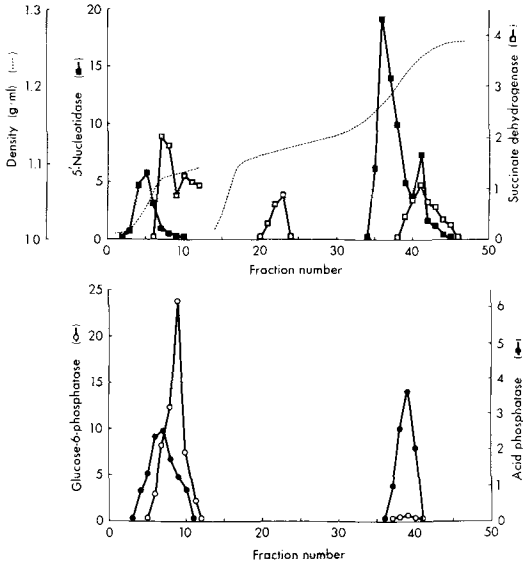

FIG. 1. Rate-isopycnic zonal fractionation of hepatic nuclear sediment. The activities of enzymes are expressed as follows: 5'-nucleotidase and glucose-6-phosphatase, μmoles inorganic phosphate released per 14 ml zonal fraction per hour; succinate dehydrogenase, μmoles 2,6-dichlorophenolindophenol reduced per 14 ml zonal fraction per hour; acid phosphatase, μmoles p-nitrophenol reduced per 14 ml zonal fraction per hour.

fatty acid methyl esters (28) was also used to aid their identification. The identification of some fatty acids was checked using a stainless steel column packed with 10% DEGS-PS on 80/100 Supelcoport (Supelco Inc., Bellefonte, PA). In the second experiment, fatty acids were analyzed using the DEGS-PS and JXR columns. The relative proportions of fatty acids were calculated from the product of the peak height and retention time of the esters on the EGSS-X or DEGS-PS column.

RESULTS AND DISCUSSION

Isolation of Plasma Membranes

A typical sedimentation profile obtained by rate-isopycnic centrifugation is shown in Figure 1. Plasma membranes were collected in a sharp peak with an average banding density from three runs of 1.18 g/ml at 20 C. This procedure resulted in a 31-fold increase in the specific activity (on a protein weight basis) of 5'-nucleotidase compared to that in a 10% homogenate of liver. The membranes were virtually free of mitochondria, lysosomes and endoplasmic reticulum. Electron microscopy of zonal fractions from several centrifuge runs also indicated a pure preparation of plasma membranes.

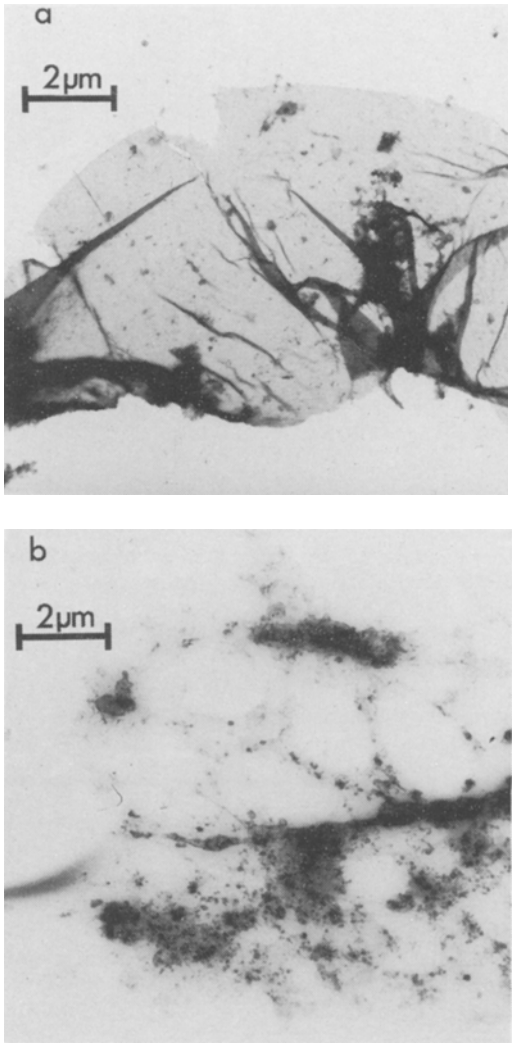

FIG. 2. Electron micrographs of plasma membranes from a zonal fraction. a. Typical plasma membrane sheet. b. Vesicular material associated with plasma membrane sheet.

Plasma membrane fractions contained large sheets of membraneous material and were free of other identifiable cellular components (Fig. 2a). In some instances, vesicular material was seen lying close to the surface of membrane sheets (Fig. 2b).

Fatty Acid Composition of Hepatocyte Plasma Membrane Lipids

The fatty acid compositions of the plasma membrane lipids from male and female mice fed the same diet were similar. The results for the male and female mice have, therefore, been pooled and the results of the first experiment are shown in Table IV. Palmitic and stearic

TABLE IV

Fatty Acid Composition of Hepatocyte Plasma Membrane Lipids from Mice Fed a Low Fat Diet or High Fat Diets Containing Either Polyunsaturated or Saturated Fat

Fatty acids	Diet: Low fat	Polyunsaturated fat			Saturated fat	
	Time (weeks)					
	0	2	4	6	8	30
12:0[a]	1.1±0.23[b]	0.6±0.06				
14:0	7.7±0.11	1.5±0.03	2.6±0.06	1.3±0.03	1.5±0.06	1.6±0.16
16:0	26.8±0.53	32.7±1.36	30.2±0.96	37.3±0.29	32.8±1.12	27.8±0.35
16:1	1.5±0.02	1.2±0.03	0.7±0.04	0.8±0.03	3.4±0.24	3.0±0.32
18:0	44.9±0.52	47.9±0.73	38.0±0.31	28.2±0.31	30.0±1.83	21.0±0.60
18:1	4.8±0.07	3.5±0.10	6.0±0.06	16.1±1.13	17.2±0.99	30.8±1.17
18:2	2.1±0.08	2.4±0.18	8.3±0.20	6.6±0.19	4.2±0.69	3.6±0.14
18:3			3.4±0.36	0.6±0.03		
20:0	6.4±0.07	5.5±0.26	0.6±0.13	1.0±0.04	3.9±0.66	3.4±1.47
20:2			Trace[c]	0.5±0.02	0.5±0.02	0.8±0.27
20:4	4.7±0.25	4.7±0.22	6.9±0.49	5.0±0.39	4.7±0.89	8.0±0.90
22:0			2.0±0.28	1.8±0.53	1.8±0.19	
22:2			1.3±0.33	0.8±0.16		
Saturates	86.9	88.2	73.4	69.6	70.0	53.8
Monoenes	6.3	4.7	6.7	16.9	20.6	33.8
Dienes	2.1	2.4	9.6	7.9	4.7	4.4
Polyenes[d]	4.7	4.7	10.3	5.6	4.7	8.0
Mean chain length[e]	17.3	17.4	17.6	17.4	17.5	17.6
Unsaturation index[f]	29.3	28.3	63.7	54.5	48.8	66.6

Fatty acids	Diet: Low fat	Saturated fat			Polyunsaturated fat	
	Time (weeks)					
	0	2	4	6	8	30
12:0	1.1±0.23					
14:0	7.7±0.11	2.8±0.11	1.0±0.04	1.6±0.06	1.6±0.03	2.9±0.05
16:0	26.8±0.53	34.0±1.45	38.9±0.26	37.6±0.98	27.4±0.59	40.2±0.15
16:1	1.5±0.02	1.2±0.04	1.0±0.02	2.5±0.46	Trace[c]	0.5±0.12
18:0	44.9±0.52	43.1±0.78	39.6±0.23	35.0±1.65	37.0±0.30	29.6±0.12
18:1	4.8±0.07	10.4±0.38	14.6±0.30	13.8±0.29	12.9±0.36	19.1±0.14
18:2	2.1±0.08	2.7±0.17	2.0±0.06	3.9±0.11	10.8±0.40	3.4±0.32
18:3				0.9±0.03	1.5±0.24	Trace[c]
20:0	6.4±0.07	2.3±0.14	0.5±0.02	1.1±0.26	1.2±0.08	1.4±0.24
20:2				Trace[c]	0.9±0.05	
20:4	4.7±0.25	3.5±0.09	2.4±0.13	3.0±0.23	6.7±0.13	2.9±0.07
22:0				0.6±0.15		
Saturates	86.9	82.2	80.0	75.9	67.2	74.1
Monoenes	6.3	11.6	15.6	16.3	12.9	19.6
Dienes	2.1	2.7	2.0	3.9	11.7	3.4
Polyenes[d]	4.7	3.5	2.4	3.9	8.2	2.9
Mean chain length[e]	17.3	17.3	17.2	17.2	17.6	17.2
Unsaturation index[f]	29.3	31.0	29.2	38.8	67.6	38.0

[a]Number of C atoms: number of double bonds.

[b]Units are mole % of total fatty acids. Proportions of fatty acids are the mean ± standard error of determinations on a pooled sample of liver from two or three female mice and a pooled sample from two or three male mice.

[c]< 0.5%.

[d]Three or more double bonds.

[e]Mean number of C atoms per fatty acid.

[f]Sum of the products of mole % and the number of double bonds per fatty acid. The index divided by 100 gives the number of double bonds per fatty acid molecule (29).

TABLE V

Fatty Acid Composition of Hepatocyte Plasma Membrane Lipids from Mice Fed a Low Fat Diet or High Fat Diet Containing Either Polyunsaturated or Saturated Fat

Fatty acids	Diet: Low fat	Polyunsaturated fat		
		Time (weeks)		
	0	2	4	8
14:0[a]	10.0±0.06[b]	6.0±0.15	1.6±0.41	4.1±0.78
16:0	31.3±0.14	24.7±0.26	21.9±0.24	32.6±0.25
16:1	1.3±0.05	1.3±0.20	2.6±0.87	0.9±0.07
18:0	25.0±1.36	31.0±1.16	31.2±0.73	40.4±0.92
18:1	12.3±0.58	9.5±0.27	8.3±0.61	8.7±0.30
18:2	7.0±1.02	8.6±0.81	14.2±0.64	4.0±0.32
18:3		Trace[c]	2.0±0.16	
20:0	7.3±0.12	9.4±1.27	4.0±0.42	4.3±0.35
20:2		2.1±0.81	2.1±0.48	Trace[c]
20:4	5.8±0.81	5.7±0.55	8.6±0.81	5.0±0.69
22:0		1.7±1.59	2.6±0.07	
22:2			0.9±0.21	
Saturates	73.6	72.8	61.3	81.4
Monoenes	13.6	10.8	10.9	9.6
Dienes	7.0	10.7	17.2	4.0
Polyenes[d]	5.8	5.7	10.6	5.0
Mean chain length[e]	17.2	17.7	17.9	17.4
Unsaturation index[f]	50.8	55.0	85.7	37.6

Fatty acids	Diet: Low fat	Saturated fat		
		Time (weeks)		
	0	2	4	8
14:0	10.0±0.06	2.0±0.38	3.8±0.92	4.0±0.35
16:0	31.3±0.14	26.9±1.30	30.2±0.56	36.1±1.40
16:1	1.3±0.05	3.1±0.81	2.3±0.18	1.3±0.05
18:0	25.0±1.36	27.8±0.54	26.8±1.19	29.9±1.33
18:1	12.3±0.58	15.4±2.59	18.1±0.98	15.7±0.42
18:2	7.0±1.02	6.8±0.45	6.4±1.04	2.1±0.10
18:3				
20:0	7.3±9.12	7.7±0.98	4.4±0.96	4.6±1.11
20:2		1.0±0.45	Trace[c]	
20:4	5.8±0.81	6.5±0.82	6.3±0.97	4.3±0.64
22:0		1.9±0.72	1.7±0.76	2.0±0.31
Saturates	73.6	67.2	66.9	76.6
Monoenes	13.6	18.5	20.4	17.0
Dienes	7.0	7.8	6.4	2.1
Polyenes[d]	5.8	6.5	6.3	4.3
Mean chain length[e]	17.2	17.7	17.5	17.4
Unsaturation index[f]	50.8	60.1	58.4	38.4

a,b,c,d,e,f See footnotes on Table IV.

acids constituted over 70% of the fatty acid in the plasma membrane lipids and the main unsaturated fatty acids were oleic, linoleic, and arachidonic acids. These have been reported (30) to be the major fatty acids in the phospholipids of mouse hepatocyte plasma membranes. The small amounts of arachidic acid in the plasma membrane lipids were probably derived from sphingomyelin (30) which is present in greater proportions in plasma membrane lipids than in the lipids of whole liver (31). There was, however, some variation in the proportions of fatty acids in the plasma membrane lipids prepared from different mice. Other workers (30) have also reported wide variation in the proportions of palmitic and stearic acids and other fatty acids which are not always present in these membrane lipids.

The proportions of linoleic and arachidonic acids were less than those reported for rat hepatocyte plasma membrane phospholipids by Keenan and Morré (32). They found a greater proportion of linoleic acid in rat hepatocyte plasma membrane phosphatidylcholine than we have observed in total lipids from mouse hepatocyte plasma membranes. However, sphingomyelin, a major plasma membrane phospholipid, contained only a trace of linoleic acid and plasma membrane triglyceride contained only small proportions of linoleic and arachidonic acids. This apparent difference in the fatty acid composition of hepatocyte plasma membrane lipids may, therefore, be due to the fact that we determined the fatty acid composition of the total lipids extracted from plasma membranes. These results thus present the weighted mean proportions of fatty acids from all lipid classes.

In order to substantiate the results of the first experiment, the effect of the diets on the fatty acid composition of hepatocyte plasma membrane lipids was examined in a second experiment (Table V). With the exception of a small proportion of lauric acid in the plasma membrane lipids of mice in the first experiment, the same fatty acids were present in hepatocyte plasma membrane lipids from mice fed the low fat diet in both experiments. There was, however, some variation in the proportions of a couple of fatty acids. In the second experiment, the plasma membrane lipids of mice fed the low fat diet contained greater proportions of oleic and linoleic acids and smaller proportions of stearic acid than in the first experiment.

In the first experiment, the plasma membrane lipids of mice fed the polyunsaturated fat diet for 4 wk contained increased proportions of the major dietary fatty acid, linoleic acid, and increased proportions of arachidonic acid. The proportions of these fatty acids declined when mice continued to receive this diet. These findings were confirmed in the second experiment. In this experiment, the proportions of linoleic and arachidonic acids reached a maximum after mice had been fed the polyunsaturated fat diet for 4 wk, but had declined after mice had received the diet for 8 wk. When mice were fed the saturated fat diet after the polyunsaturated fat diet, the proportion of linoleic acid also declined. The proportion of oleic acid in the plasma membrane lipids increased when mice in the first experiment were fed either of the high fat diets. In the second experiment, this increase was observed only when mice were fed the saturated fat diet, possibly because of the shorter time scale of the second experiment and the higher proportion of oleic acid in the saturated fat diet than in the polyunsaturated fat diet.

The mean carbon chain lengths and molar unsaturation indices of the fatty acids present in the plasma membrane lipids from mice in the first experiment are shown in Table IV and those for mice in the second experiment are shown in Table V. Small increases in the mean carbon chain length were observed when the mice were fed the high fat diets. The increases observed when the mice were fed the polyunsaturated fat diet were due to the increased proportions of linoleic and arachidonic acids, while those observed when the mice were fed the saturated fat diet were due to the increased proportion of oleic acid. In the first experiment, the unsaturation index increased when mice were fed the polyunsaturated fat diet for 4 wk. With continued feeding of the polyunsaturated fat diet, the unsaturation index declined, but by week 30 the index was still higher than that when mice were fed the low fat diet. This was due to the increased proportion of oleic acid in plasma membrane lipids at week 30. The results of the second experiment confirmed that a transient increase in unsaturation of plasma membrane lipids occurred when mice were fed the polyunsaturated fat diet. At week 30, the increased proportion of oleic acid in the plasma membrane lipids of mice fed the saturated fat diet was the main cause of the increased unsaturation of these membranes (Table IV). At this point, the plasma membrane lipids of mice fed the saturated fat diet were more unsaturated than those of mice fed the polyunsaturated fat diet for the same time.

It has now been conclusively demonstrated that increases in the proportion of fat in the diet lead to decreases in the de novo synthesis of fatty acids (33-35). It is likely, therefore,

that most of the fatty acid in the hepatocyte plasma membranes of the mice fed the high fat diets reported here would have been derived from the dietary long chain fatty acids and not from acetyl CoA. The dietary fatty acids could have been incorporated unmodified or modified by elongation, desaturation, or both of these processes (36). The increased proportion of arachidonic acid present when mice were fed the polyunsaturated fat diet was, therefore, presumably formed by desaturation and elongation of the dietary linoleic acid. It is also interesting that the proportion of oleic acid in the plasma membrane lipids increased when mice were fed either of the high fat diets, while there was only a transient increase in the proportion of linoleic acid when mice were fed the polyunsaturated fat diet. However, the biochemical explanation for this transient increase is not known.

The increased unsaturation of the plasma membrane lipids reported here indicate that physical properties of the plasma membranes, such as permeability, may have changed (see Introduction). However, further studies should examine the diet-induced changes in the fatty acid composition of the different lipid classes as lipid-induced alterations in membrane function are thought to be determined mainly by the membrane phospholipids (4).

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Fatty Acids of Cerebrosides in Different Regions of the Developing Foetal Brain

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ABSTRACT

The fatty acid composition of cerebrosides of developing foetal human brain and regional variations, if any, during intrauterine life were studied. While palmitic and stearic acids were the predominant normal fatty acids throughout intrauterine life, long chain fatty acids, like lignoceric and nervonic acids, which were low at early gestational ages, rapidly accumulated at term. Regional differences were observed in the concentrations of long chain normal fatty acids especially at term. Medulla oblongata showed a greater accumulation of long chain fatty acids as compared to the cerebellum and cerebrum. The distribution of 2-hydroxy fatty acids in different regions showed a pattern predominantly that of long chain carbon units, even at 34 wk of foetal life. A higher ratio of lignoeric to stearic acid in the case of normal fatty acids, probably indicative of chain elongation, was also evident in the case of medulla oblongata and cerebellum as compared to the cerebrum. The significance of these qualitative alterations in relation to rapid growth of brain prior to term and the process of myelination has been discussed.

INTRODUCTION

Maturation of brain is associated with the process of myelination and cerebrosides constitute important lipid fractions of myelin. In fact, a sharp rise in the concentration of cerebrosides is taken as an indicator of myelination (1). It has earlier been observed that the fatty acid composition of cerebrosides of brains of adult subjects is different from that of brains of infants (2). There is, however, little information on the fatty acid composition of cerebrosides of developing human brain during intrauterine life and regional variations, if any. It is important to obtain such information in view of the well documented reports that undernutrition during early stages of life can adversely affect the composition of lipids in the brain (3,4). Results of a study, wherein the fatty acid profile of cerebrosides of the developing human foetal brain was determined, are presented here.

MATERIALS AND METHODS

All foetuses were obtained from the maternity ward of a teaching hospital. These were obtained from apparently normal mothers belonging to the poor socio-economic group, who underwent medical termination of pregnancy. Foetuses which were stillborn and where the cause of death was nonneurological were also included. Foetuses with congenital abnormalities and those born to mothers who had suffered from any complication during pregnancy were excluded.

The gestational ages of foetuses ranged from 20 wk to full term. Gestational age was determined from the last menstrual period (LMP). Information about LMP was gathered by careful questioning of mothers by relating the data to specific events with the use of a local calendar. Data thus obtained have been found to be correct to within ± 7 days (5). Foetuses with unknown LMP were rejected.

Foetuses were obtained within 4 hr after death, brains removed and dissected into the three major anatomical regions—cerebrum, cerebellum, and medulla oblongata. In a small number of foetuses, where immediate dissection was not possible, the whole foetus was stored at -20 C. All brain samples were frozen and stored at -20 C until taken for analyses. In all, 22 brains were analyzed for the fatty acid composition of cerebrosides.

Isolation of Cerebrosides

A 20% homogenate of brain was prepared in glass distilled water for all brain samples. A suitable aliquot of the homogenate was used for extraction of lipids using chloroform and methanol, 2:1 following the procedure of Folch et al. (6). Glycolipids were isolated from total lipids by silicic acid chromatography (7). Pure cerebrosides were later separated by thin layer chromatography from the total glycolipid fraction using a solvent system comprising chloroform and methanol, 100:20, and Silica Gel G. This system ensured complete separation of cerebrosides from other lipid constituents. On the chromatograms, lipid fractions were

TABLE I

Fatty Acid Composition of Cerebrosides in Cerebrum of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation											
	12:0	14:0	16:0	18:0	18:1	20:0	20:1	22:0	22:1	24:0	24:1	Others[a]
	Percentage of total methyl esters											
20-22 (4)[b]	4.5[c]	4.5	19.3	30.5	2.5	5.1	1.9	5.5	3.3	4.5	3.3	10.4
24-26 (3)	4.0	6.3	20.2	31.8	7.5	4.4	5.4	1.3	4.9	6.9	3.1	4.0
30-32 (3)	5.1	7.3	19.6	40.2	5.0	1.5	2.5	5.7	3.2	3.0	1.9	1.8
34-36 (5)	3.1	3.3	23.4	44.5	1.9	1.6	4.3	2.5	0.7	4.1	4.3	4.5
Term (7)	2.7	2.6	29.1	32.3	1.1	1.6	3.7	1.7	0.8	7.0	10.0	4.6

[a]Include fatty acids with retention time equivalent to 16:1, 17:0, 19:0, 19:1, 23:0, 23:1 or higher than that of 24:1.

[b]Figures in parentheses indicate number of brains analyzed.

[c]All values are mean values.

TABLE II

Fatty Acid Composition of Cerebrosides in the Cerebellum of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation											
	12:0	14:0	16:0	18:0	18:1	20:0	20:1	22:0	22:1	24:0	24:1	Others[a]
	Percentage of total methyl esters											
20-22 (3)	3.1	3.9	31.8	22.3	2.3	4.7	1.8	14.7	5.8	1.4	0.8	4.8
24-26 (3)	4.5	3.3	19.8	20.9	5.7	3.7	2.7	1.9	12.4	8.6	7.4	5.0
30-32 (4)	4.1	4.6	13.5	21.6	5.2	7.5	5.8	7.8	1.7	9.2	3.1	12.4
34-36 (5)	6.0	3.9	21.0	31.7	4.4	2.0	0.8	3.5	1.8	10.9	6.6	4.4
Term (5)	1.3	3.2	13.6	19.2	1.7	3.4	2.8	10.2	16.1	17.5	5.1	3.2

[a]Please see legends under Table I.

detected by exposure to iodine vapors. Dried chromatoplates were placed in a closed glass chamber of 10 liter capacity containing 1 g of iodine crystals at its bottom. The exposure of the plate to iodine vapors for 10 to 15 sec was sufficient to detect lipid bands. Standard bovine cerebrosides were run simultaneously. Under these conditions, distinct double spots characteristic of cerebrosides with Rf of 0.55 were evident. Sulphatides and phospholipids separated out with lower Rf values. Areas corresponding to cerebrosides were scraped off from the plate. Four 5 ml portions of chloroform-methanol, 2:1, followed by two 5 ml portions of acetone were used to ensure complete elution of cerebrosides from Silica Gel G.

Preparation of Methyl Esters of Fatty Acids and Their Analyses

Purified samples of cerebrosides were taken in stoppered glass tubes and dissolved in 0.2 ml of benzene and subjected to methanolysis following the procedure adopted by Stallberg-Stenhagen and Svennerholm (8). To the samples contained in glass tubes, 5 ml of 5% (w/v) methanolic hydrochloric acid was added. The tubes were tightly stoppered after flushing with N_2 gas and kept at 80 C for 16 hr. The reaction mixture, after addition of 10 ml of distilled water, was extracted three times, with 5 ml portions of petroleum ether each time. The pooled petroleum ether extracts were rinsed with 5 ml of 5% (w/v) solution of sodium bicarbonate and later twice with 5 ml portions of distilled water. The extracts were dried over anhydrous sodium sulphate. The resulting methyl esters which contained normal fatty acid methyl esters and small proportions of 2-OH fatty acid methyl esters were subjected to thin layer chromatography. A solvent system comprising petroleum ether, ether, and acetic acid (90:10:1) was used.

Unsubstituted and 2-OH fatty acid methyl esters were identified by brief exposure to iodine vapors as described earlier. A controlled experiment with bovine cerebrosides before and after exposure to iodine under these conditions revealed that exposure to iodine vapors not exceeding 15 sec had little affect on the concentration of any fatty acid ($\Delta = \angle 0.5\%$).

Unsubstituted fatty acid methyl esters were eluted with chloroform-methanol (9:1) mixture, while 2-OH fatty acid methyl esters were eluted with diethyl ether. Normal fatty acid methyl esters were taken in chloroform

TABLE III

Fatty Acid Composition of Cerebrosides in Medulla Oblongata of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation											
	12:0	14:0	16:0	18:0	18:1	20:0	20:1	22:0	22:1	24:0	24:1	Others[a]
	Percentage of total methyl esters											
20-22 (3)	4.7	4.6	25.7	33.8	2.6	5.3	4.2	4.9	1.4	7.1	2.6	5.2
24-26 (2)	4.1	4.3	24.9	18.0	3.1	3.9	2.9	10.5	1.6	8.3	6.5	8.9
30-32 (2)	3.2	5.1	19.8	24.7	5.3	3.9	1.0	5.6	2.5	16.3	5.3	5.9
34-36 (5)	4.0	6.4	16.4	34.8	1.1	2.7	1.3	6.9	0.2	10.6	4.7	5.5
Term (6)	2.5	2.7	17.5	13.5	1.0	2.1	1.1	14.8	4.4	20.0	6.6	11.4

[a]Please see legends under Table I.

TABLE IV

2-Hydroxy Fatty Acids of the Cerebrosides in the Cerebrum of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation[a]						
	h 16:0	h 18:0	h 20:0	h 22:0	h 23:0	h 24:0	h 24:1
	Percentage of total trimethylsilyl derivatives of fatty acid methyl esters						
30-32 (2)[b]	10.4[c]	16.2	6.0	32.9	2.7	24.2	7.4
34-36 (2)	1.6	2.5	5.3	14.2	5.1	56.9	16.0
Term (6)	4.3	7.2	1.7	26.1	2.7	44.4	10.2

[a]Fatty acids constituting less than 1% were excluded.

[b]Figures in parentheses indicate number of brains analyzed.

[c]All values are mean values.

and subjected to gas chromatography on 15% polydiethylene glycol succinate, coated on 80-100 mesh Chromasorb-W; silanized 2-hydroxy fatty acids were converted to their respective trimethyl silyl ethers by treating with a mixture of pyridine, hexamethyl disilazane, and trimethyl chlorosilane in the proportion of 0.5:0.1 and 0.05 ml each, respectively. Trimethyl silyl ethers were chromatographed on S.E.-30 columns. Wilkens Model 650 Aerograph fitted with flame ionization detector was used. The carrier gas was nitrogen with a flow rate of 25 ml/min and the oven temperature was 200 C in both the cases.

Standard fatty acid methyl esters were obtained from Sigma Chemical Co., St. Louis, MO and Applied Science Laboratories, State College, PA. Individual 2-hydroxy fatty acid methyl esters were identified by comparing retention data with those of purified acids (kindly made available by Dr. J.F. Mead, Department of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, CA).

RESULTS AND DISCUSSION

The fatty acid patterns of cerebrosides in the three different regions of the brain are presented in Tables I, II and III. Mean values represented in tables are percentages of total methyl esters of fatty acids.

Palmitic (16:0) and stearic (18:0) acids formed the major fatty acids of cerebrosides at all gestational ages in all the three regions of the brain. Between the gestational ages of 20 wk and term, values for these two fatty acids together ranged from 52 to 68% in the case of cerebrum, 33 to 54% and 31 to 60% in the case of cerebellum and medulla oblongata, respectively. The concentrations were relatively higher in cerebrum as compared to the other two regions. The values did not alter much with increasing period of gestation in the cerebrum. However, values in cerebellum and medulla oblongata showed a decreasing trend towards term. The reduction was mostly due to a fall in the palmitic acid content in the cerebellum, while in the medulla oblongata both palmitic and stearic acids showed a decreasing trend. At term, values for these fatty acids in cerebrum, cerebellum, and medulla oblongata were 61.4, 32.8, and 31%, respectively. As a result, at term in the cerebrum, longer chain fatty acids constituted a lower proportion as compared to the other two regions.

Lignoceric (24:0) and nervonic (24:1) acids showed, in general, an increasing trend towards term in all regions. The concentration of lignoceric acid was maximal at term as compared to

TABLE V

2-Hydroxy Fatty Acids of the Cerebrosides of the Cerebellum of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation						
	h 16:0	h 18:0	h 20:0	h 22:0	h 23:0	h 24:0	h 24:1
	Percentage of total trimethylsilyl derivatives of fatty acid methyl esters						
30-32 (2)	40.8	7.4	4.1	25.5	5.1	10.4	6.0
34-36 (2)	1.6	17.2	2.4	20.9	5.9	38.9	14.7
Term (6)	5.1	9.8	13.3	13.7	7.3	38.8	9.7

[a]Please see legends under Table IV.

TABLE VI

2-Hydroxy Fatty Acids of Cerebrosides in the Medulla Oblongata of Developing Human Brain

Gestational age in weeks	Fatty acid carbon number and unsaturation[a]						
	h 16:0	h 18:0	h 20:0	h 22:0	h 23:0	h 24:0	h 24:1
	Percentage of total trimethylsilyl derivatives of fatty acid methyl esters						
30-32 (2)	39.8	8.7	3.2	26.4	3.4	10.2	3.7
34-36 (2)	1.4	2.8	14.2	29.4	2.8	48.3	3.3
Term (6)	2.0	5.6	6.5	16.7	4.7	48.8	11.3

[a]Please see legends under Table IV.

TABLE VII

Ratio of Lignoceric (24:0) to Stearic (18:0) Acid in Cerebrosides from Different Regions of the Developing Brain[a]

Gestational age in weeks	Cerebrum	Cerebellum	Medulla Oblongata
20-22	0.19[b]	0.07	0.21
24-26	0.19	0.70	0.46
30-32	0.07	0.36	0.72
34-36	0.10	0.50	0.33
Term	0.26	1.00	1.55

[a]Number of samples analyzed for these regions is the same as indicated in Tables I, II and III.

earlier gestational ages, irrespective of the region. Certain inconsistencies in the trend of various fatty acids (i.e., 20:0, 20:1, 22:0, 22:1 and also 24:0 and 24:1 to certain extent) were, however, noticeable between the gestational ages 24 and 36 wk. Alterations in the concentration of these fatty acids did not follow any set pattern with increasing gestational age.

At term, lignoceric and nervonic acids together constituted 17.0% in the cerebrum, 22.6% in the cerebellum, and 26.6% in the case of the medulla oblongata. Since lignoceric and nervonic acids constitute the major long chain fatty acids of myelin, the increase in their concentration with advancing gestation may be considered as reflecting an increase in myelin. Further, long chain fatty acids are believed to contribute to membrane stability by virtue of their interdigitation (9).

The distribution of 2-hydroxy fatty acids of cerebrosides in different brain regions is depicted in Tables IV, V and VI. At earlier gestational ages, the relative proportion of hydroxy fatty acids was too low for satisfactory analysis. Hence, quantitation of the fatty acid species were confined to the latter part of intrauterine life, i.e., 30 wk to term. Fatty acids above 19 carbon units constituted more than 80% of total hydroxy derivatives after 34 wk of gestational age in all the three regions studied. Their concentration at 30 wk was, however, lower and different in different regions (cerebrum = 73%; cerebellum = 51%, and medulla oblongata = 46%). Unlike normal fatty acids, the pattern of distribution of 2-hydroxy species was predominantly that of long chain carbon units—especially after 34 wk. Cerebronic acid (hydroxy 24:0 = h 24:0)

formed the major constituent after 34 wk of intrauterine life in all the regions of the brain, but at 30 wk of gestation, its concentration was considerably lower, especially in cerebellum and medulla oblongata. The lower levels of h 24:0 were associated with elevated concentrations of 2-hydroxy palmitic acid (h 16:0) in these regions.

The significance of elevated levels of cerebronic acid at early stages of life is not known. The gradual increase of lignoceric (24:0) acid with the progress of gestation, however, coincides with the maturity and the development of the brain and thus, lignoceric rather than cerebronic acid might serve as better indicator of myelin deposition.

In Table VII are given the relative ratios of lignoceric to stearic acid (24:0/18:0) for the three regions of the brain. It is believed that this ratio reflects activities of the enzymes involved in the chain elongation process. The ratio was well below 1.0 in all the three regions of the brain between 20 and 36 wk of intrauterine life. In general, an increasing trend in this ratio was observed with progress of gestation for cerebellum and medulla oblongata, although there were certain inconsistencies around 22-24 wk of gestation in the former and around 34-36 wk in the latter case. At term, this ratio in cerebellum and medulla oblongata was 1.00, 1.55, respectively, values which are four and sixfold higher than in the cerebrum which had a ratio of 0.26. Since lignoceric acid is eventually formed from stearic acid, these data indicate that there are regional differences in the brain with respect to the rate at which this conversion occurs.

The higher concentration of lignoceric acid may mean greater myelination and earlier maturation of the cerebellum and medulla oblongata as compared to the cerebrum. Even at lower gestational ages, medulla oblongata showed relatively higher values for this ratio because of increased concentration of lignoceric acid, indicating an earlier myelination of this region prior to birth. These observations are in line with the results obtained from studies on rat brain by Smith (10) who observed that the hind brain matures earlier and that development of myelin proceeds rostrally from the hind brain to the cerebrum.

A relatively significant increase in long chain fatty acids a few weeks prior to birth corresponds to remarkable increase in several chemical constituents of brain, viz. nucleic acids, protein, and lipids (11,12) and also polyenoic fatty acid of phospholipids (13). Thus, data presented here on the fatty acid composition of cerebrosides and data reported earlier on the fatty acid profile of phospholipids (13) in human brain indicate that qualitative changes in terms of alterations associated with fatty acids synchronize with quantitative deposition of chemical constituents following brain growth spurt. Regional difference in the rate of myelination and maturation of the brain might depend on these primary biochemical events.

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Analysis of 4-Methyloctanoic Acid and Other Medium Chain-Length Fatty Acid Constituents of Ovine Tissue Lipids

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ABSTRACT

Medium chain-length (C_6-C_9) fatty acids in depot, intramuscular, kidney, and liver lipids from pasture-fed sheep and subcutaneous and intramuscular lipids from barley-fed sheep were analyzed with emphasis on the 4-methyloctanoic acid (hircinoic acid) content. Within individual animals, the level of hircinoic acid in the subcutaneous fat was usually higher than that either in the deeper depot fats or in the intramuscular fat. Variation of hircinoic acid levels between animals was greatest in the subcutaneous fat and least in the perinephric fat. There were usually higher levels of both branched and odd carbon chain fatty acids in the lipids of barley-fed sheep than in the corresponding lipids of pasture-fed sheep.

INTRODUCTION

A number of medium chain-length (C_6-C_{10}) fatty acids have been identified as constituents of mutton fat (1), some having been implicated as contributors to mutton flavor (2,3). 4-Methyloctanoic acid (hircinoic acid), in particular, is considered to be primarily responsible for the "sweaty" flavor note in mutton and goat meat (3).

It is generally recognized that the fatty acid composition of triglycerides differs according to its anatomical disposition in the body (4), and information on the variation of higher fatty acids ($>C_{10}$) between different ovine tissue fats is available (5-8). This information has been extended in the present paper, in which we present results of studies on the variation in medium chain-length fatty acid composition of lipids from different tissues of individual sheep. A comparison has also been made between the hircinoic acid composition of lipids from similar anatomical sites in pasture-fed and barley-fed animals.

Because of the low concentration levels (total $<0.1\%$) and the relative volatility of these compounds, they are not normally revealed by the conventional methods of fatty acid analysis (9). This paper describes a method for the quantitative chemical analysis of hircinoic acid and of other medium chain-length volatile acid constituents in mutton fat, developed for use in connection with comparative studies on the organoleptic qualities of different mutton samples.

MATERIALS AND METHODS

Animals

The sheep used in this study were Suffolk x (Border Leicester x Romney) crosses. Four wethers and two rams were pasture-fed (perennial ryegrass–white clover mixture). A further three wethers were pasture-fed to 30 kg live weight after which they were fed ad libitum barley-containing pellets (71% barley, 20% lucerne meal, 2% corn oil, 2% molasses, and 5% vitamin-mineral mixture). The animals were slaughtered at ca. 50 kg live weight when 7-11 mo old.

Isolation of Fatty Acids

Lipids were extracted from samples of adipose tissue (subcutaneous, perinephric, and omental fats) as described previously (3). Minced lean meat (200 g), liver or kidney was homogenized with chloroform/methanol (2:1 v/v, 600 ml, 2 x 300 ml) and the extract filtered through Celite 545. The filtrate was shaken with an equal volume of distilled water, the chloroform layer separated, and the solvent removed under reduced pressure.

Lipid samples (2 g) were saponified and the medium chain-length fatty acids concentrated by a single steam distillation-diethyl ether extraction as described previously (3), except that 2-methyloctanoic acid (ca. 20 μg) was used as an internal standard instead of nonanoic acid. The ethereal extract was passed through a column containing a mixture of potassium chloride (15 g) and anhydrous sodium carbonate (5 g). Nonacidic components were eluted with diethyl ether (100 ml) after which nitrogen was passed through the column. The dried

powder from the column was dissolved in water (50 ml). Diethyl ether (20 ml) was added followed by sulfuric acid (25% v/v, 25 ml) in small portions with careful stirring. The ethereal layer was separated and the aqueous layer extracted twice more with 20 ml portions of ether. The combined ether extracts were washed with a saturated potassium chloride solution (40 ml), dried over anhydrous sodium sulfate, and carefully taken to dryness.

Fatty acids in the extracts were esterified by refluxing with methanolic boron trichloride (1 ml) for 15 min in a 10 ml conical flask. Pentane (2 ml) was added to the cooled reaction mixture followed by saturated potassium chloride solution until the organic layer was in the neck of the flask. The pentane phase was transferred to a 1 ml Reacti-vial (Pierce Chemical Co., Rockford, IL) and reduced to 0.2 ml under a stream of nitrogen.

Gas Chromatography and Mass Spectrometry

Fatty acid methyl esters were analyzed on a 3.7 m x 3.2 mm OD stainless steel column containing 10% w/w silicone OV-101 on 100-120 mesh Gas Chrom Q (Applied Science Labs., State College, PA). The column was constructed for backflushing, as shown in Figure 1, in a Hewlett-Packard model 7620A gas chromatograph. A "tee-piece" on injection port "A" was connected to the inlet end of the column and to an on-off valve located outside the oven. Half way along the column, another "tee-piece" was inserted and connected to injection port "B." The column outlet was connected to the flame ionization detector.

During normal operation of the column, the valve was in the "off" position and the flow of carrier gas through injection port "A" was 40 ml/min and through injection port "B" was 5 ml/min. Ester samples (ca. 2 μl of ethereal solution) were introduced into injection port "A" and the oven temperature programmed from 100 C at 2 C/min. After compounds of interest had emerged, the program rate was increased to 20 C/min, the valve was carefully opened and the carrier gas through injection port "B" was increased to 100 ml/min. These conditions were maintained for 10 min after the maximum oven temperature (220 C) had been reached, after which normal operating conditions were resumed. Concentrations of hircinoic acid and of the other acids, compared with the internal standard, were measured with a Hewlett-Packard model 3380A integrator.

Shorter nonsplit columns (2.5 m x 3.2 mm) containing either 10% w/w silicone OV-101 or 10% w/w stabilized polyethylene glycol adipate (EGA, Analabs, North Haven, CT) on 100-120 mesh Gas Chrom Q were also used. These were usually programmed over the temperature range 50-200 C at 2 C/min. Plots of equivalent chain length vs. emergence temperature of normal saturated fatty acid or methyl ester peaks were linear over most of the temperature program range that was used.

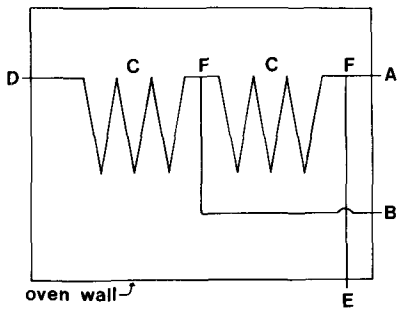

FIG. 1. Arrangement of gas chromatography column in oven for backflushing. A, B = injection ports, C = two halves of column, D = detector, E = on-off valve, F = "tee-pieces."

Mass spectra were recorded at 70 ev with an AEI MS-30 mass spectrometer that was coupled via a silicone elastomer membrane interface to a Pye 104 gas chromatograph. Helium (flow rate 40 ml/min) was used as the carrier gas.

Preparation of 2-Methyloctanoic Acid

The methanesulfonate of 2-octanol was prepared in 85% yield by the method of Crossland and Servis (10). It was purified by column chromatography through Florisil using petroleum ether as the eluting solvent. The methanesulfonate (10 g) was converted to the nitrile by refluxing with sodium cyanide (10 g) and Aliquat 336 (1 g, methyltricaprylylammonium chloride, General Mills Chem. Inc., Kankakee, IL) in acetonitrile (20 ml) for 2 hr. Petroleum ether (20 ml) was then added followed by water (40 ml). The mixture was shaken and the upper layer removed, dried over anhydrous sodium sulfate, and the solvent removed under reduced pressure. Yield of nitrile was 6.0 g (96%). The nitrile (4.2 g) was hydrolyzed by refluxing in ethylene glycol (24 ml) containing potassium hydroxide (6.7 g) for 3 hr. Water (24 ml) was added and the reaction mixture extracted with diethyl ether (20 ml, 2 x 10 ml). The combined ethereal extracts were washed with water (2 x 10 ml) and discarded. The combined aqueous extracts were acidified with sulfuric acid (25% v/v) and extracted with diethyl ether (3 x 20 ml) which was subsequently dried over anhydrous sodium sulfate and then removed under reduced pressure. Yield of acid was 2.0 g (43.3%).

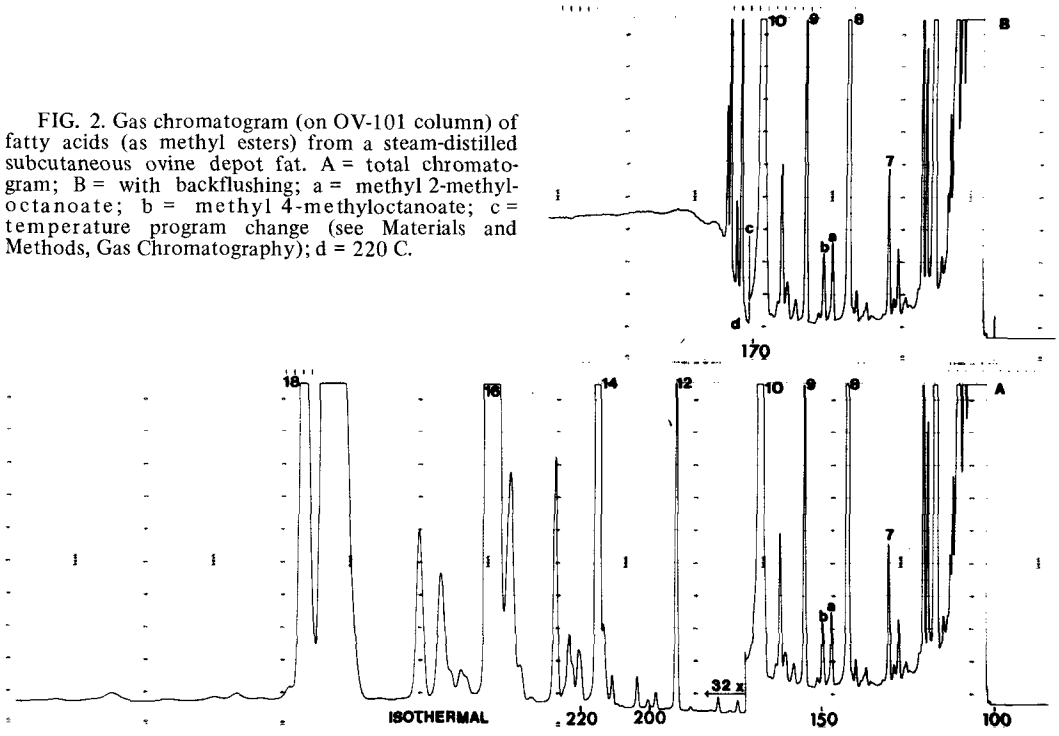

FIG. 2. Gas chromatogram (on OV-101 column) of fatty acids (as methyl esters) from a steam-distilled subcutaneous ovine depot fat. A = total chromatogram; B = with backflushing; a = methyl 2-methyloctanoate; b = methyl 4-methyloctanoate; c = temperature program change (see Materials and Methods, Gas Chromatography); d = 220 C.

The acid and the corresponding methyl ester each gave a single peak when subjected to analysis by gas chromatography. Equivalent chain lengths of the methyl ester on the EGA and OV-101 columns were 8.0 and 8.4, respectively, and that of the acid on the EGA column was 8.1. The mass spectrum of the methyl ester had peaks at m/e 88 (relative intensity, 100%), M-15 (0.8%), M-31 (2.8%) and M-59 (1.1%), typical of this type of compound. A series of peaks corresponding to loss of hydrocarbon fragments from the molecular ion (m/e 172, 1.1%) were present at m/e 143 ($M-C_2H_5$, 2.0%), 129 ($M-C_3H_7$, 2.8%), 115 ($M-C_4H_9$, 3.9%), and 101 ($M-C_5H_{11}$, 23%). There was no peak at m/e 74.

Preparation of 4-Methyloctanoic Acid

This acid was prepared by chain extension of butyl bromide according to the following method. Diethyl butylmethylmalonate was prepared in 88% yield from butyl bromide, diethyl methylmalonate, sodium hydride, and Aliquat 336 by the phase transfer method of Durst and Liebeskind (11). The malonate was decarboxylated to a mixture of ethyl and glycol monoesters in 95% yield by refluxing it initially in ethanolic potassium hydroxide (0.5 mol potassium hydroxide to 1.0 mol malonate) for 1 hr followed by removal of the ethanol under reduced pressure, then a further 2 hr reflux in ethylene glycol. A sample (ca. 0.5 g) of the monoesters was converted to the methyl ester by refluxing in boron trichloride/methanol (1 ml) for 30 min, while the balance was reduced by lithium aluminium hydride to 2-methylhexanol (12) (91% yield). The alcohol was converted into its methanesulfonate in 84% yield (10) and this was chain-extended to diethyl (2-methylhexyl)-malonate (80%) (11) and thence, to the corresponding ethyl and glycol monoester mixture (85%) by the methods described above. Saponification of the esters yielded 4-methyloctanoic acid.

The acid and the corresponding methyl ester each gave a single peak when subjected to analysis by gas chromatography. Equivalent chain lengths of the methyl ester on the EGA and OV-101 columns were 8.5 and 8.6, respectively, and that of the acid on the EGA column was 8.6. The mass spectrum of the methyl ester had peaks at m/e 74 (relative intensity 88%), M-15 (0.5%) and M-31 (15%), typical of this type of compound. Peaks locating the methyl branch were present at m/e 87 (100%), 85 (3.9%) and 115 (29%). Other peaks in the high mass region were at m/e 172 (molecular ion, 0.5%), 143 ($m-C_2H_5$, 5.0%), 125 (2.0%) and 123 (5.0%). This spectrum was similar to that previously reported (2).

RESULTS

A typical total gas chromatogram of the methyl esters from the steam-volatile acids of a subcutaneous mutton fat sample is shown in Figure 2.

The backflushing gas chromatographic procedure (upper chromatogram in Fig. 2) effectively removed the need for a second distillation-extraction step (3) and reduced the gas chromatographic analysis time from 2 hr to 45 min. More than 150 analyses were performed before the column showed signs of deterioration (tailing of peaks).

Unfortunately, methyl 2-methyloctanoate was not suitable as an internal standard for use with the EGA column. Separation of either 2-methyloctanoic acid and octanoic acid or of the corresponding methyl esters was not possible on the EGA column. Good separation of the methyl esters of these acids, as well as those of 4-methyloctanoic, 6-methyloctanoic and nonanoic acid was achieved on the longer silicone column. The nonpolar liquid phase was therefore found to be suitable for the analysis of methyl hircinoate with methyl 2-methyloctanoate as the added (internal) standard.

The time required for complete esterification of 2-methyloctanoic acid was 10 min whereas only 2 min was required when normal acids were esterified. Steric hindrance caused by the proximity of the methyl branch to the carboxyl group probably caused this time difference. Unless compounds other than the nonanoic acids were being analyzed, weight/area gas chromatographic corrections were not necessary. These corrections were usually much less than the variation of repeated analyses of individual samples. Variations of up to 20% for hircinoic acid values in the individual fats sample analyses were considered satisfactory for the current investigation. Efficiency of collection of compounds varied from 50% to 100% based on the standard ester counts recorded on the integrator. If less than 50% was collected, the result was rejected.

A calibration plot (Fig. 3) was prepared by adding known amounts of synthetic hircinoic acid to a mutton perinephric fat sample that had a low value for this acid and analyzing the mixture by the above method. This plot gives an indication of the range of values received from four distillations at each concentration level of hircinoic acid.

Repeated analyses (four of each) of four subcutaneous mutton fat samples gave hircinoic acid values (ppm ± SD) of 9.2 ± 1.0, 8.0 ± 0.6, 5.4 ± 0.6, and 6.2 ± 1.0. Repeated gas chromatographic analyses on another single distillate gave hircinoic acid values (ppm) of 12.1, 11.0, 10.4, and 11.4.

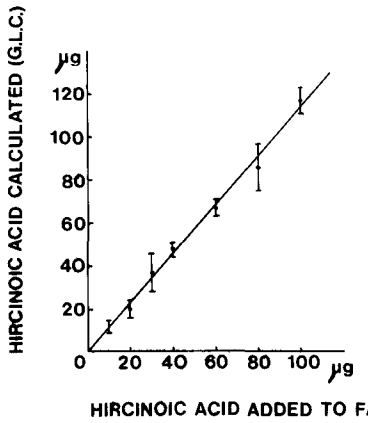

FIG. 3. Hircinoic acid analysis of a perinephric fat containing various amounts of added hircinoic acid, with allowance made for the natural concentration of this acid in the fat. Least squares line of regression with means and standard deviations.

Concentrations of the normal, iso and anteiso fatty acids (C_6-C_9) and of hircinoic acid in the lipids of various adipose tissues, lean meats, and organs are given in Tables I-III.

Assessment of differences between the means was made using analysis of variance and least significant difference (LSD) methods (13). Log values were used in order to reduce the variability of the standard deviations between the depots. Assessment of the repeatability of the ranking of the six sheep over five sites (subcutaneous fat, perinephric fat, and intramuscular fat from the shoulder, loin, and leg) was made using Kendall's Coefficient of Concordance (W) (14).

DISCUSSION

Results in Table I show that, in general, the hircinoic acid level is higher in the subcutaneous fat than in the internal depot fats, with the largest variation between animals being manifest also in the subcutaneous fat. The perinephric fats from different animals on the other hand show consistent low values for hircinoic acid. There was a small but significant difference ($P<0.001$) in the hircinoic acid levels between the perinephric and omental fats.

In the intramuscular fats (Table II), hircinoic acid values all fall within the range 2.8 ± 1.0 ppm. These values in most instances are lower than that for the subcutaneous fat of the same animal. There was considerable variability in the ranking of six sheep for hircinoic acid levels in fat from the five different sites with a Coeffi-

TABLE I

Concentration (in ppm) of Medium Chain Fatty Acid Constituents in Depot Fats of Sheep[a]

Depot fat	Animal[c]	Acid[b]							
		n-6	ai-7 (4-Me-7)	n-7	i-8 (6-Me-8)	n-8	ai-9 (6-Me-9)	n-9	HA (4-Me-9)
Subcutaneous brisket	R1	10	0.9	3.9	1.5	44	0.4	9	6.2
	R2	14	0.2	4.8	1.8	51	0.4	7	2.3
	W44	16	0.9	2.5	1.0	50	0.3	8	2.5
	W40	10	1.0	4.8	0.9	44	1.0	11	9.0
	W15	13	0.3	5.0	0.9	83	0.4	17	14.2
	W62	15	1.5	8.4	1.8	103	0.5	17	19.4
	Mean ± SD	13 ± 3[x]	0.8 ± 0.5	4.9 ± 2.8[x]	1.3 ± 0.4	63 ± 25[x]	0.5 ± 0.3	12 ± 4[x]	8.9 ± 6.8[x]
Subcutaneous loin	B8	22	26	22	19.2	168	7.2	100	48.2
	B38	24	14	16	8.4	218	7.0	96	63.2
	B58	22	17	17	9.2	220	10.9	129	65.0
	Mean ± SD	23 ± 1	19 ± 6	18 ± 3	12.3 ± 6.0	202 ± 29	8.4 ± 2.2	108 ± 18	59 ± 9
Perinephric	R1	13	0.6	1.8	0.3	66	0.2	9	1.4
	R2	36	0.7	4.9	1.1	78	0.5	13	2.0
	W44	24	1.9	3.7	0.7	78	0.2	7	1.6
	W40	28	0.8	5.9	1.0	89	0.3	15	1.8
	W15	37	0.7	3.6	0.7	73	0.7	13	1.4
	W62	24	0.7	3.2	0.6	120	0.6	16	1.7
	Mean ± SD	27 ± 9[y]	0.9 ± 0.5	3.9 ± 1.4[x]	0.7 ± 0.3	84 ± 19[x,z]	0.4 ± 0.2	12 ± 3[x]	1.7 ± 0.2[y]
Omental	R1	20	1.0	3.0	0.6	88	0.4	11	3.7
	R2	36	0.7	9.1	0.2	80	0.6	22	2.8
	W44	27	1.5	2.9	0.2	98	0.6	10	2.3
	W15	23	0.9	5.5	0.4	97	0.4	16	3.3
	W62	32	1.4	4.7	0.5	163	0.4	18	4.2
	Mean ± SD	28 ± 7[y]	1.1 ± 0.3	5.0 ± 2.5[x]	0.4 ± 0.2	105 ± 33[x]	0.5 ± 0.1	15 ± 5[x]	3.3 ± 0.7[z]

[a]Values in the same column of the normal acids and of hircinoic acid without a common superscript are significantly different ($P<0.05$).

[b]n = normal, i = iso, ai = anteiso. 4-Me-7 = 4-Methylhexanoic acid. 6-Me-8 = 6-Methylheptanoic acid. 6-Me-9 = 6-Methyloctanoic acid. 4-Me-9 = 4-Methyloctanoic acid. HA = hircinoic acid.

[c]R = ram, w = wether, B = barley-fed wether.

TABLE II

Concentration (in ppm) of Medium Chain Fatty Acid Constituents in Intramuscular Lipids of Sheep[a]

Intramuscular	Animal[c]		Acid[b]								
			% Fat	n-6	ai-7 (4-Me-7)	n-7	i-8 (6-Me-8)	n-8	ai-9 (6-Me-9)	n-9	HA (4-Me-9)
Shoulder	R1		16.8	61	1.4	30.0	1.1	179	0.2	31	1.9
	R2		8.9	16	0.6	9.6	2.0	82	0.2	15	4.5
	W44		17.1	80	1.3	25.1	0.9	128	0.2	26	2.1
	W40		12.4	9	0.9	9.7	2.3	70	0.2	11	2.3
	W15		14.6	14	0.1	15.1	2.1	101	0.2	15	2.2
	W62		15.7	17	1.3	7.8	0.7	145	0.5	31	3.9
		Mean ± SD	14.3 ± 3.1	33 ± 30^{x,y}	0.9 ± 0.5	16.2 ± 9.2^{y}	1.5 ± 0.7	118 ± 41^{y,z}	0.2	22 ± 9^{y}	2.8 ± 1.1^{y,z}
Leg	R1		4.5	36	1.5	23.5	0.5	93	1.0	24	1.2
	R2		6.0	32	0.3	12.9	1.7	58	0.3	25	1.6
	W44		10.0	9	1.5	9.1	1.1	51	0.8	7	4.8
	W40		8.5	41	0.5	21.4	1.3	79	0.5	27	2.1
	W15		6.5	27	1.1	11.2	0.4	69	1.6	28	4.6
	W62		5.1	29	0.5	11.5	1.3	103	0.4	26	2.8
		Mean ± SD	6.8 ± 2.1	29 ± 11^{y}	0.9 ± 0.5	14.9 ± 6.0^{y}	1.1 ± 0.5	76 ± 20^{x}	0.8 ± 0.5	23 ± 8^{y}	2.9 ± 1.5^{y,z}
Loin	R1		4.1	47	3.9	27.5	0.7	148	0.9	33	2.0
	R2		5.7	42	0.7	18.7	2.3	94	0.7	31	2.2
	W44		9.1	59	2.0	8.2	0.3	257	1.2	43	1.8
	W40		7.6	32	0.3	18.1	1.5	89	0.4	23	1.8
	W15		6.4	38	0.4	14.8	1.8	94	1.5	27	3.0
	W62		4.3	30	0.2	15.7	1.1	141	0.5	27	2.8
		Mean ± SD	6.2 ± 1.9	41 ± 11^{y}	1.3 ± 1.5	17.2 ± 11.8^{y}	1.3 ± 0.7	137 ± 64^{y}	1.0 ± 0.5	31 ± 7^{y}	2.3 ± 0.5^{y,z}
	B8		4.7	38	2.0	17.8	0.5	211	1.0	50	4.9
	B38		5.5	44	3.6	16.3	0.5	218	0.7	44	6.4
	B58		7.8	46	7.8	18.7	1.4	186	1.7	47	10.7
		Mean ± SD	6.0 ± 1.6	42 ± 4	4.5 ± 3.0	17.6 ± 1.2	0.8 ± 0.5	205 ± 17	1.1 ± 0.5	47 ± 3	7.3 ± 3.0

[a]Values in the same column of the normal acids and of hircinoic acid without a common superscript are significantly different ($P<0.05$).

[b]n = normal, i = iso, ai = anteiso, 4-Me-7 = 4-Methylhexanoic acid, 6-Me-8 = 6-Methylheptanoic acid, 6-Me-9 = 6-Methyloctanoic acid, 4-Me-9 = 4-Methyloctanoic acid, HA = hircinoic acid.

[c]R = ram, W = Wether, B = barley-fed wether.

TABLE III

Concentration (in ppm) of Medium Chain Fatty Acid Constituents in Kidney and Liver Lipids of Sheep

Organ fat	Animal[b]		Acid[a]								
			% Fat	n-6	ai-7 (4-Me-7)	n-7	i-8 (6-Me-8)	n-8	ai-9 (6-Me-9)	n-9	HA (4-Me-9)
Kidney	R1		2.9	6	0.6	4.6	1.3	26	0.6	8	1.5
	R2		2.2	26	0.4	10.2	1.3	44	0.4	22	3.0
	W44		1.9	10	0.8	7.2	0.8	52	7.0	12	4.2
	W15		3.8	23	0.3	12.3	1.4	50	0.3	28	1.1
	W62		4.3	44	0.2	17.9	1.1	88	0.5	36	2.0
		Mean ± SD	3.0 ± 1.0	22 ± 15	0.5 ± 0.2	10.4 ± 5.1	1.2 ± 0.2	52 ± 23	1.8 ± 2.9	21 ± 12	2.4 ± 1.3
Liver	R1		3.6	5	0.8	7.9	1.7	24	2.3	11	2.3
	R2		3.1	17	1.5	11.0	2.5	19	2.0	17	2.0
	W44		5.0	8	1.7	12.0	3.6	59	1.6	15	1.6
	W40		3.9	56	1.7	39.7	7.3	86	1.8	64	1.8
		Mean ± SD	3.9 ± 0.8	22 ± 24	1.4 ± 0.4	17.7 ± 14.8	3.8 ± 2.5	47 ± 45	1.9 ± 0.8	27 ± 25	1.9 ± 0.3

[a]n = normal, i = iso, ai = anteiso, 4-Me-7 = 4-Methylhexanoic acid, 6-Me-8 = 6-Methylheptanoic acid, 6-Me-9 = 6-Methyloctanoic acid, 4-Me-9 = 4-Methyloctanoic acid, HA = hircinoic acid.

[b]R = ram, W = wether, B = barley-fed wether.

cient of Concordance value of 0.29 ($P>0.05$).

The contrast between subcutaneous and intramuscular fats is greatly magnified in the case of the barley-fed animals. Hircinoic acid levels in both types of fats in these animals are higher than corresponding values for the pasture-fed animals. Likewise, whereas the medium chain-length iso and anteiso acids were present in only trace amounts in lipids from pasture-fed animals, greatly elevated values of these acids and also odd chain-length acids were found in the subcutaneous fat of the barley-fed animals. This variation was not so apparent in the intramuscular lipids. The feeding of barley (and of other cereals) (15) to sheep has been previously shown to result in an increase of the levels of other branched-chain fatty acids, mostly in the subcutaneous fat (16-18).

Levels of the normal acids were usually significantly higher in the intramuscular lipids than in the depot fats (Tables I and II). Kidney and liver lipids (Table III) had similar overall fatty acid compositions, in the range studied, to those of the intramuscular lipids (Table II).

Results in Tables I-III on the whole indicate that, within any particular animal, the medium chain-length fatty acid composition in the lipids from the different tissues is quite variable, in line with previous findings for higher fatty acids.

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A Structural Model for the Cholesterol-Phosphatidylcholine Complexes in Bilayer Membranes

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ABSTRACT

Based on the structural properties of phospholipid and cholesterol molecules, and making use of the known structural and motional effects of cholesterol and its analogs on phospholipid bilayers, a model for the cholesterol-phosphatidylcholine complex is proposed. In this model, the 3β-hydroxyl group of cholesterol is assumed to engage in hydrogen bonding with the carbonyl oxygen of the fatty acyl groups in phospholipids. Some specific configurations of the saturated and unsaturated fatty acyl chains of the phospholipid are suggested to participate in van der Waals attractive interactions with the α and β surface of the steroid nucleus.

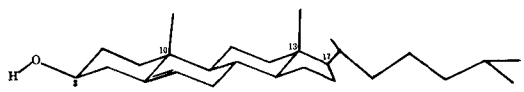
INTRODUCTION

In recent years, many advances have been made in establishing the effects of cholesterol on the structural and motional properties of phospholipid acyl chains (1-7). Based on the results of these studies, it is now widely accepted that the cholesterol-phospholipid interaction involves primarily the hydrophobic group interactions. In addition, the effect of cholesterol on phospholipids in bilayers appears simply to restrict differentially the number of rotomers accessible to the segments of the fatty acyl chains. However, relatively little systematic work has been carried out toward understanding the effect of cholesterol headgroup on phospholipid membranes. Thus, unlike most other studies on cholesterol-phospholipid interactions which have focused primarily on the nonpolar region of the complex, we have investigated the effect of cholesterol on the properties of the phospholipid bilayer to include the interactions of the polar groups with one another and with water (8-12).

In this communication, a structural model for the phospholipid-cholesterol complex that accounts for the interactions of both hydrophobic and hydrophilic groups between these amphiphilic molecules is proposed and some specific time-averaged configurations of cholesterol and phospholipid in the interacting complex are suggested. Since most membrane phospholipids consist of two fatty acyl chains with the saturated acid to be localized at position 1 and the unsaturated acid at position 2 of the glycerol moiety, and since large numbers of studies on the cholesterol-phospholipid interaction have been carried out with egg phosphatidylcholine, the cholesterol-phospholipid interaction discussed here is limited to mixed natural phosphatidylcholine such as egg phosphatidylcholine or synthetic phosphatidylcholine which contains a saturated acid in position 1 and an unsaturated acid in position 2. The proposed model is based on the structural properties of cholesterol and phosphatidylcholine and on some experimental evidence concerning the effect of cholesterol on phosphatidylcholine. A comparison between this model and other models for the cholesterol-phosphatidylcholine complex is included.

Structural Properties of Cholesterol

X-ray studies have shown that the overall dimension of cholesterol is 7.2 x 5 x 20 Å (13). Structurally, it is characterized by the three groups shown in the following figure (I).



I

(a) 3β-OH is a relatively small hydrophilic group covalently attached to the carbon 3 of the steroid nucleus.

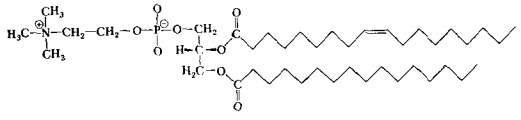
(b) The steroid nucleus is a fused tetracyclic ring system that is hydrophobic and sterochemically rigid. The length of the fused rigid ring system is 9 Å. The CPK space-filling molecular model shows that the α surface of the fused ring system is planar but the β surface is puckered due to the presence of two angular methyl groups at C_{10} and C_{13}. It was observed by nuclear magnetic resonance (NMR) that the rigid steroid nucleus is oriented parallel to the hydrocarbon chains of [$C_{16:0}$-$C_{16:0}$] dipalmitoylphosphatidylcholine in bilayers (14).

(c) A branched, extended hydrocarbon side chain attached to C_{17} of the rigid ring system is

another nonpolar group. This side chain is known from various NMR studies to exhibit a high degree of mobility in the phospholipid bilayer (6,7,15).

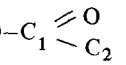
Structural Properties of Phosphatidylcholine

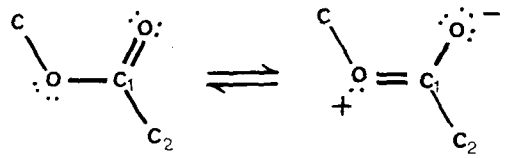
From the structural formula (II), it is also possible to subdivide the phosphatidylcholine molecule into three distinct groups.



II

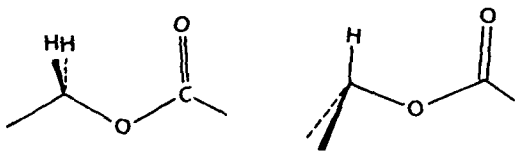
(a) $-CH_2-O-PO_2^--O-CH_2-CH_2-{}^+N(CH_3)_3$ is the polar headgroup of the molecule. X-ray data suggest that the preferred orientation of this group in the *crystal* is that in which the dipolar axis of the zwitterion is parallel to the hydrocarbon chain (16). In egg phosphatidylcholine bilayer, however, the ^{31}P NMR experiments suggests a bent configuration of the zwitterion with the terminal $^+N(CH_3)_3$ group rotating rapidly in the vicinity of the negatively charged phosphate group (11). This bent configuration is further supported by neutron scattering data (17).

(b) The small segment taken up by the structural element  is termed the interface region, since it links the polar headgroup and fatty acyl chains of phospholipids. Here C_1 and C_2 are, respectively, the first and second carbon atoms of the fatty acyl chain. This structural element has the following resonance forms:



III IV

Participation of canonical form IV imparts significant double bond character to the C_1-O bond. Hence, all the carbon and oxygen atoms in the interface region are planar, and the carbonyl oxygen is held relatively rigid in proximity to the hydrocarbon chain (12). Mathieson (18) has shown that for secondary esters the conformation VI is invariably adopted. For primary esters, the evidence points to V as the preferred conformation. The orientation of the C–H bond relative to the C_1=O bond is, therefore, not equivalent at the two chains in the interface region.



V VI

It should be noted that the carbonyl oxygen atom in the interface region can serve as a hydrogen bond acceptor, but the water molecule may not have the necessary orientation to serve as the hydrogen bond donor here (12).

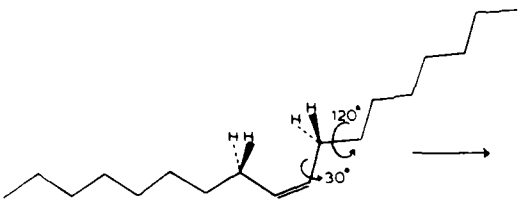
(c) The fatty acyl chains constitute the third region of the phosphatidylcholine molecule. These groups are strongly hydrophobic. For egg phosphatidylcholine molecules, the chain located at position 1 of the glycerol backbone contains predominantly 16:0 (32%) and 18:0 (16%) fatty acids, and the other chain contains predominantly 18:1 (31%), 18:2 (16%), and minor amounts of 16:1 and 20:4 fatty acids. If we discount the first two carbons which are parts of the structural element in the interface region, the hydrocarbon chains are still at least 14 CH_2-unit long. However, due to the selective presence of carbon-carbon double bonds, structural properties of the two long fatty acyl chains are different.

The preferred configuration of the long saturated fatty acyl chain at carbon position 1 is a straight chain in bilayers because the linear configuration can interact most favorably with other acyl chains (19). However, the carbon-carbon single bond in the saturated chain does undergo rapid *trans-gauche* isomerization. The energy barrier for such rotation is about 3.5 Kcal mol^{-1} (20). In membranes, the rotation may not simply tarry at any fixed carbon position, but may migrate up and down the hydrocarbon chain. In addition, several *gauche* (g) conformations may be present at any instant of time. In order to maintain the preferred linear configuration, β-coupled *trans-gauche* isomerizations probably occur in the bilayer (21). For instance, when rotations about two carbon-carbon single bonds occur in opposite directions, such as g^+ and g^-, and they are separated by one carbon-carbon bond in *trans* (t) conformation, the resulting hydrocarbon chain will be laterally displaced, but retain its linear configuration (22,23). These g^+tg^- and g^-tg^+ kinks, or β-coupled configuration, will create free

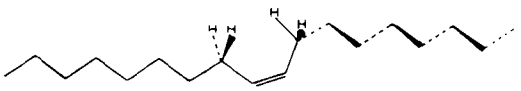
volumes of hydrophobic pockets; the chain, however, is shortened in length by the equivalent of one CH_2 unit length.

The presence of a carbon-carbon double bond in the second fatty acyl group increases the probability of internal molecular rotations around the carbon-carbon single bonds in the hydrocarbon chains. As discussed earlier, the energy barrier separating a *trans* conformation from a *gauche* conformation is about 3.5 Kcal mol^{-1}. The presence of a double bond, however, reduces the energy barrier for the adjacent C-C single bond rotations to about 2 Kcal mol^{-1} (20). Results obtained by ^1H- and ^{13}C-NMR studies on phosphatidylcholine molecules with varying degrees of unsaturation are indeed compatible with the notion that an unsaturated acyl chain is intrinsically more mobile than a saturated one (24-27).

For the second chain located at the 2 position of the glycerol backbond, the introduction of a carbon-carbon *cis* double bond (Δ) between C_9 and C_{10} into an *all trans* C-18 hydrocarbon chain will result in a bent configuration which is sterically restricted by the neighboring saturated chain (Figure 1a). However, if a single *gauche* rotation occurs at the β position on either side of the *cis* double bond and the adjacent α carbon rotates about the C=C–C single bond by 30°, then the overall configuration of this unsaturated chain will become linear but laterally displaced (Figure 1b and c); that is,



VII



VIII

As discussed above, the unsaturated chain is intrinsically more flexible, hence, it is expected that the Δtg kink may exist. Raman studies of Lippert and Peticolas (28) indicate that *gauche* conformations are present on both sides of the double bond in dioleolyllecithin. Moreover, Batchelor *et al.* (29) have found that the carbon atom β to a *cis* double bond has a relatively higher probability of a *guache* conformation. These results strongly suggest the existence of Δtg kink. Thus, the $C_{18:1}$ chain of phospholipids in the bilayer appears most likely to have two linear and parallel straight segments linked by a Δtg kink. The length of each segment depends on the position of the *gauche* conformation. If the *gauche* conformation occurs at the C_7-C_8 single bond, then the first linear segment is $\sim$ 7.5 Å in length (Figure 1b). On the other hand, when the *trans-gauche* isomerization occurs at the C_{11}–C_{12} single bond, the first all-*trans* linear segment extends down to C_9 and has a length of $\sim$10 Å (Figure 1c). For the convenience of later discussion, we define the long molecular axis of the chain as the Z-axis and the axis along which lateral displacement of the chain occurs due to the Δtg kink-formation as the Y-axis (Figure 1). It can be seen that the angular methyl group of cholesterol can fit excellently into the free volume of the hydrophobic pocket generated by the kink formation. However, only the $\Delta_{9,10}$tg, not the g$t\Delta_{9,10}$, kink has the necessary geometry and dimensions to fit the packing requirements of the two angular methyl groups on the β-side of the cholesterol steroid nucleus. We shall discuss these requirements later. Since it is now well known that the order parameter of the last three carbons including the methyl carbon of the saturated fatty acyl chain is quite low (30,31) and that $\sim$18% of the egg phosphatidylcholine unsaturated acyl chain has a carbon-carbon unsaturated double bond located between C_{12} and C_{13}, the lower hydrophobic volume formed by the $\Delta_{9,10}$tg kink near the center of egg phosphatidylcholine bilayer may not exist at all due to the chain disorder in that region. It is worth mentioning that the branched alkyl side chain of cholesterol exhibits high degrees of mobility (6,7,15). This mobile portion of the molecule can fit isotropically in the disordered portion of the fatty acyl chains.

Cholesterol Phosphatidylcholine Bilayers

Many studies have been carried out in the past to investigate the bilayer properties affected by the introduction of cholesterol. Evidence for the existence of molecular interaction between cholesterol and phospholipids in bilayers is manifold [for recent review, see Jain (32)]. Here, I selectively summarize some data published in the literature concerning the effect of cholesterol on the bilayers which are composed of either egg phosphatidylcholine or synthetic phosphatidylcholine molecules con-

taining a saturated and an unsaturated fatty acyl chains.

(a) X-ray diffraction analysis shows cholesterol to have a condensing effect on egg phosphatidylcholine bilayers (33-36). Fourier analysis of X-ray diffraction pattern of egg phosphatidylcholine-cholesterol bilayers shows a marked localization of the acyl terminal methyl groups in the center of the bilayer. These data suggest that addition of cholesterol reduces the lipid chain motions so that the chains are well oriented with their terminal groups localized (34).

(b) ^{1}H-NMR studies have shown that cholesterol has a differential effect on the phosphatidylcholine mobility (37,38). The magnitudes and linewideths of N-methyl proton resonances are little changed; the linewidth of methylene proton signals is affected much more than the resonance of the terminal methyl protons. This selective line-broadening effect of cholesterol implies that the cholesterol molecules affect the fatty acyl chain motions without disturbing the polar headgroup. Furthermore, it implies that cholesterol has little direct effect on the mobility of the terminal methyl group of the hydrocarbon chain. ^{2}H-NMR data also show that the presence of cholesterol in egg phosphatidylcholine bilayer vesicle has no effect on the quadrupole splittings and relaxation rates for ^{2}H in the choline methyl group, in contrast to its profound effect on the spectra for ^{2}H resonances of the hydrocarbon chains (39).

(c) The ^{13}C-NMR spectra of egg phosphatidylcholine and egg phosphatidylcholine-cholesterol bilayers in molar ratios of 2:1 and 1:1 have been reported (40,41). As with ^{1}H- and ^{2}H-NMR results described above, selective effects of cholesterol on egg phosphatidylcholine carbon signals have been observed with ^{13}C-NMR, the largest influence occurring in the center portion of the fatty acyl chains. In addition, ^{13}C-NMR spectra show that the olefinic carbon resonances are markedly decreased in amplitude and broadened upon addition of cholesterol. This additional information implies that C_9 and C_{10} of the unsaturated fatty acyl chain are most likely in direct van der Waals contact with the steroid nucleus of cholesterol in such a way that the olefinic carbons have less motional freedom than the acyl terminal methyl group. Despite the selective line-broadening effect, the spin-lattice relaxation time, T_1, of individual carbon atoms along the fatty acyl chain in egg phosphatidylcholine bilayers is nearly unchanged by the addition of 50 mole % cholesterol (41). It is interpreted that cholesterol has little effect on the rapid, isolated or localized *trans-gauche* isomerizations

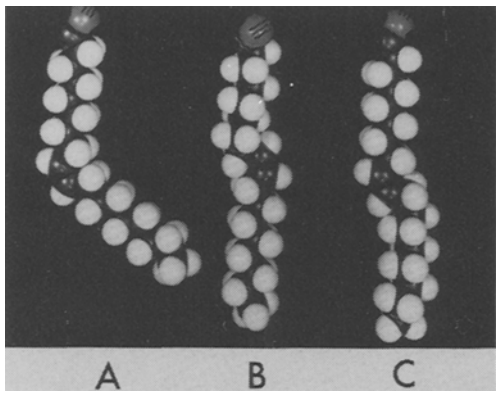


FIG. 1. Corey-Pauling-Koltun space-filling models of the $C_{18:1}$ fatty acyl chain of natural phosphatidylcholine. (A) An all-*trans* conformation with a *cis* $\Delta_{9,10}$ double bond. (B) The gt$\Delta_{9,10}$ kink, where g represents a *gauche* conformation between C_7 and C_8 of the chain. (C) The $\Delta_{9,10}$tg kink, where g represents a *gauche* conformation between C_{11} and C_{12} of the chain.

of the fatty acyl chains but, rather severely restricts the long-range swinging motion (T_2*) of the carbons in the central region of lipid hydrocarbon chains.

(d) It has been shown by the analysis of the Raman bands for the carbon-carbon stretching vibrations near 1100 cm^{-1} region that the contribution of *gauche* conformations is decreased upon cholesterol addition (5). Since the ^{13}C-NMR relaxation times, T_1, of the carbon atoms along the fatty acyl chain is not affected within experimental resolution by cholesterol and since the value of T_1 probably represents the localized higher frequency *trans-gauche* isomerizations of the fatty acyl chains, the Raman data may thus imply that the *coupled trans-gauche* rotations is bilayers are inhibited by cholesterol.

(e) Freeze-fracture electron microscopy has demonstrated that as the bilayer sample is quenched from 23 C, cholesterol interacts preferentially with the liquid-crystalline [$C_{16:0}$–$C_{18:1}$] phosphatidylcholine in an equimolar mixture of [$C_{16:0}$–$C_{18:1}$] and [$C_{16:0}$–$C_{16:0}$] phosphatidylcholine species (42). Since the values of T_m for [$C_{16:0}$–$C_{18:1}$] and [$C_{16:0}$–$C_{16:0}$] phosphatidylcholine molecules are 0 C and 41 C, respectively, it is thus concluded that cholesterol interacts preferentially with the lower melting species upon cooling the mixture. An interesting, albeit unanswered, question is why is the rigid steroid nucleus interacting preferentially with the lower melting species.

(f) More recently, a ^{31}P$\{^{1}$H$\}$ nuclear over-

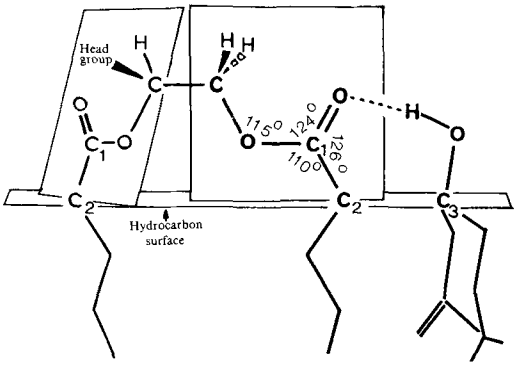


FIG. 2. Schematic diagram showing the interface region of the bilayer, composed of phosphatidylcholine and cholesterol molecules. The planarity of the structural element $-COC_1OC_2-$ of phospholipid molecule, and the proposed hydrogen-bond formation between the cholesterol 3β-hydroxyl group and the phospholipid carbonyl group are drawn diagrammatically.

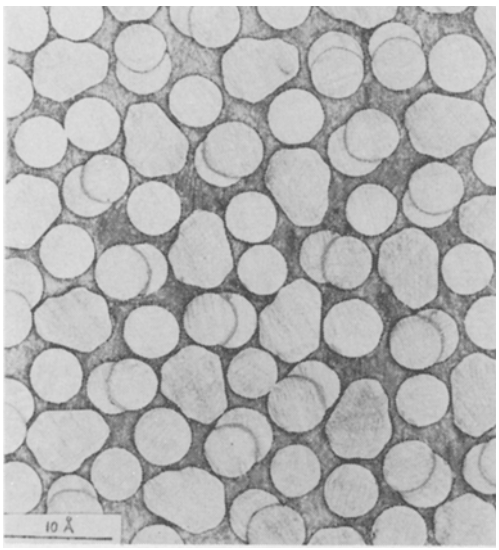


FIG. 3. Plausible arrangements of cholesterol-egg phosphatidylcholine complexes in a two-dimensional surface as projected down the long molecular axis (Z) of the lipids. The spheres, overlapped spheres, and irregular shapes represent the saturated fatty acyl chains, unsaturated acyl chains, and cholesterol molecules respectively. The proposed model suggests that the saturated chain is preferentially facing the flattened (α) side of the steroid nucleus of cholesterol molecule, while the unsaturated chain is oriented with its XZ molecular surface facing the other (β) side of cholesterol molecule. In this picture, the cholesterol molecules are randomly oriented about their long axes in the bilayer. The shapes of the saturated fatty acyl chains and cholesterol molecules are adapted from Engelman and Rothman (2).

hauser effect (NOE) has been observed in the ^{31}P-NMR spectra of egg phosphatidylcholine in bilayers. Addition of up to 30 mole % cholesterol has no effect on the NOE, T_1, or chemical shift of the phosphorus resonance (11). These results suggest that the cholesterol OH group is not interacting with the phosphate group of the phospholipid.

(g) The water binding capacity of egg phosphatidylcholine bilayers is affected by cholesterol as shown by a variety of techniques (35, 43-45). For example, the hydration (moles of water per mole of phospholipid) is 6.5 for the hydrophilic headgroup of pure egg phosphatidylcholine vesicles, whereas for egg phosphatidylcholine vesicles containing 30 mole % cholesterol the hydration increases to 9.0 (45).

(h) Lastly, it should be emphasized that carbonyl oxygens at the bilayer interface may play important roles in cholesterol-phospholipid interaction (12,46). For example, 1-oleoyl-2-palmitylglycerylphosphorylcholine and 1-oleoyl-2-palmityl-2-deoxyglycerylphosphorylcholine are phosphatidylcholine analogs which lack the C=O group in the 2 position. The condensing effect of cholesterol on these lipids in the air-water monolayer was observed to account for only one-half of the effect that was achieved on $[C_{18:1}-C_{16:0}]$ phosphatidylcholine molecules (47). Significant differences between diether- and diesterphosphatidylcholine in their interactions with cholesterol have recently been reported; the data support the conjecture that an interaction occurs between the carbonyl oxygens of diesterphosphatidylcholine and the β-OH group of cholesterol (48). Moreover, T_1 relaxation data measured by ^{13}C-NMR show that the largest effect of cholesterol on egg phosphatidylcholine molecules in vesicle bilayers as probed by a C_5 nitroxide label occurs at the fatty acyl carbonyl position (41). Finally, in the unsonicated egg phosphatidylcholine bilayers, a 2 ppm downfield chemical shift was measured for the carbonyl ^{13}C carbon in the presence of cholesterol (40), which strongly suggests hydrogen bonding of the cholesterol to the carbonyl group of the egg phosphatidylcholine molecules in bilayers (11).

The Structural Model for Cholesterol-Phosphatidylcholine Complexes

Based on the experimental evidence presented above, we can construct a model for the phospholipid-cholesterol complex in bilayer membranes.

(a) I shall begin with the interface region. *The equatorial hydroxyl group on C_3 of cholesterol molecules is proposed to hydrogen bond*

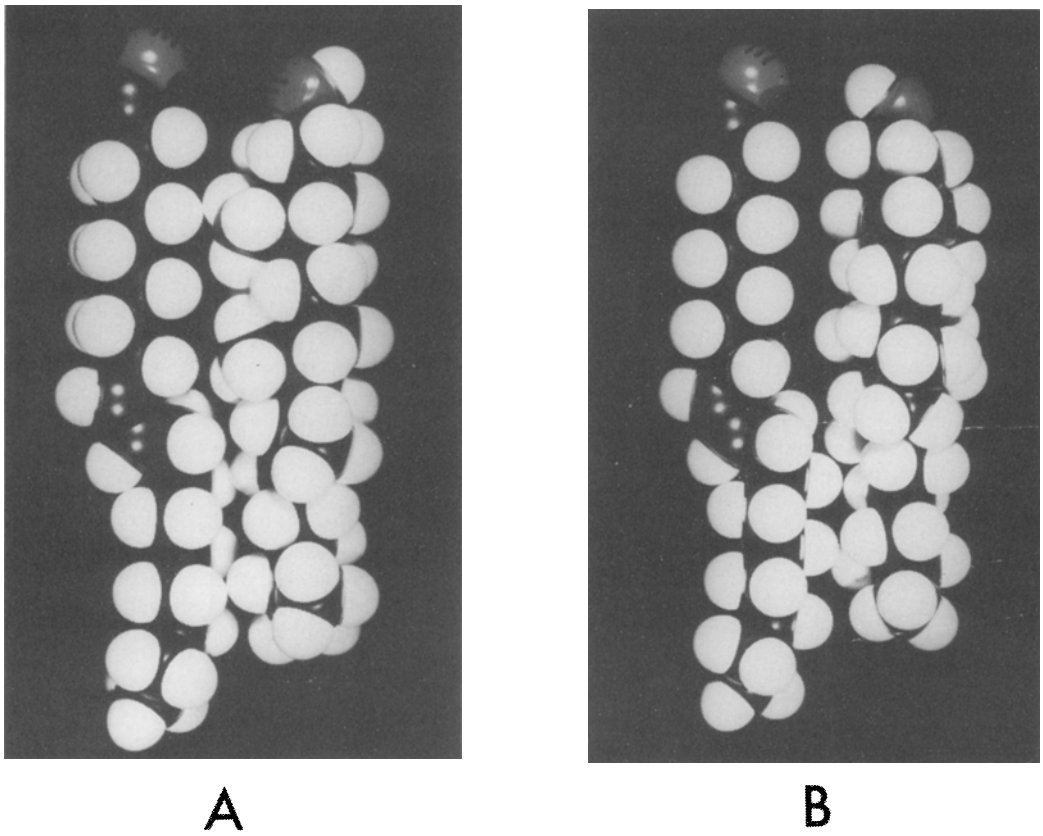


FIG. 4. CPK space-filling models of the [$C_{16:0}$–$C_{18:1}$] phosphatidylcholine and cholesterol molecule. (A) The β-axial methyl groups of cholesterol molecule are shown to fit nicely into the upper hydrophobic pocket generated by the $\Delta_{9,10}$tg kink formation in the unsaturated fatty acyl chain. (B) Molecular models show that, due to the steric hindrance of the $\Delta_{9,10}$tg kink, the α surface of the cholesterol molecule cannot be packed in van der Waal close contact with the XZ molecular surface of the upper segment of the unsaturated fatty acyl chain.

to the ester carbonyl oxygen of phosphatidylcholine molecules in bilayers (Figure 2). In this model, the sterol is so positioned that the configuration of OH...O hydrogen bond angle is ca. 15° from the linear configuration, and that the OH vector is rotated about the C_3–O bond to lie in the plane of the structural element of phospholipids ($C\diagdown O\text{–}C_1\overset{O}{\underset{C_2}{\diagup\!\!=}}$). Such structural arrangements provide a stable hydroben bond (12). This places C_3 of cholesterol at approximately the same level as C_2 of the fatty acyl chain in bilayer; hence, the steroid nucleus and fatty acyl chains are in position to participate in van der Waals type attractions for each other. The proposed model predicts that the motion of the choline headgroup in bilayers should not be restricted by cholesterol addition. Evidence abundantly supports the prediction (11, 17, 26, 38-41).

(b) The proposed model suggests that the *β-surface of the steroid nucleus is packed in close contact to the unsaturated fatty acyl chain of phosphatidylcholine molecule, close enough for the two angular methyl groups to fit into the hydrophobic pocket generated by the Δtg kink (Figures 3 and 4a).* As discussed earlier, the unsaturated fatty acyl chain has a greater stability with the gauche conformation β to *cis* double bond, and coupled isomerization such as:

$$\begin{array}{ccc} tt\Delta_{9,10}tg & \longleftrightarrow & gt\Delta_{9,10}tt \\ & \searrow \quad \swarrow & \\ & gt\Delta_{9,10}tg & \end{array}$$

may readily occur, where $\Delta_{9,10}$ represents the position of a *cis* double bond between carbon 9 and 10. The $gt\Delta_{9,10}tg$ isomer has a relatively bent configuration, while the other isomers yield two linear and asymmetric hydrophobic

pockets. The length of the upper linear segment starting from the carbonyl carbon down to the kink position is 10 and 7.5 Å for $tt\Delta_{9,10}tg$ (Figure 1c) and $gt\Delta_{9,10}tt$ (Figure 1b), respectively. The distance between the C_{18}, C_{19} angular methyl groups and the carbon-3 of the steroid nucleus can be measured from the CPK molecular model bo be 7.5 and 3 Å, respectively. In the proposed model, C_3 of cholesterol is positioned at about the same level as C_2 of the fatty acyl chain; the C_{18} angular methyl group is thus about 8.75 Å away from the carbonyl carbon of the phospholipid. Therefore, one can postulate that the two angular methyl groups can fit simultaneously only the hydrophobic pocket which has a length of 10 Å. Because of the intimate contact between the upper part of the unsaturated fatty acyl chain and the two angular methyl groups of cholesterol, van der Waals interactions would drive the folowing equilibria:

$$gt\Delta_{9,10}tg \rightleftarrows tt\Delta_{9,10}tg \rightleftarrows gt\Delta_{9,10}tt$$

toward the right, thereby decreasing the contribution of *gauche* conformation in egg phosphatidylcholine fatty acyl chains upon cholesterol addition. Moreover, dimensional analysis shows that the *cis* double bond between C_9 and C_{10} of the chain is packed next to the steroid nucleus (Figure 4a); hence, motion of the olefinic carbons is sterically hindered. This steric effect and the reduced $gt\Delta_{9,10}tt \leftrightarrow tt\Delta_{9,10}tg$ isomerization are likely to be responsible for the observed decreased in long-range swinging motions as represented by T_2* relaxation times of the carbon atoms in the center portion of the fatty acyl chains.

If, on the other hand, the β-face of the steroid nucleus is assumed to pack closer to the saturated fatty acyl chain at position 1, the protruding angular methyl groups would make the upper linear segment of the saturated chain vulnerable to conformational change. This change can only be accomplished by kink formations through rotation of single bonds from *trans* to *gauche* conformations. The experimental evidence from Raman data clearly shows that this cannot be the case, since the addition of cholesterol appears to decrease the *gauche/trans* ratio in egg phosphatidylcholine bilayers (5).

(c) *The α-face of the steroid nucleus is proposed to preferentially pack in close contact with the saturated fatty acyl chain of the phosphatidylcholine molecule (Figure 3).* Because of the planar structure of the α-surface of the steroid nucleus, it is evident that the α-plane sterically permits strong van der Waals interactions with the linear 9-carbon segment of the saturated fatty acyl chain extending from carbon 2 to carbon 10.

In the unsaturated fatty acyl chain, the lateral displacement due to kink formation is defined to occur along the Y molecular axis, and the kink is usually formed around the $\Delta_{9,10}$ *cis* double bond such as $gt\Delta_{9,10}tt$ and $tt\Delta_{9,10}tg$. Therefore, the upper part of the unsaturated fatty acyl chain with its molecular XZ plane cannot approach the α-surface of the steroid nucleus very closely (Figure 4b). The molecular YZ plane of the unsaturated fatty acyl chain, however, appears to be roughly planar. Consequently, the YZ surfaces of the unsaturated fatty acyl chains can participate in van der Waals interactions with the α-surface of the steroid nuclues. Since the unsaturated fatty acyl chains are capable of van der Waals attractive interactions with both the α- and β-surfaces of the steroid nucleus, and since the total number of unsaturated fatty acyl chains in egg phospholipids is equal to that of saturated fatty acyl chains, it is likely, therefore, that more saturated acyl chains than unsaturated acyl chains interact with the α-side of cholesterol.

Thus, according to the proposed model, simple argument will lead one to predict that phospholipids with an unsaturated fatty acyl chain will have a higher probability of interacting with the overall structure of the steroid nucleus, which includes both α- and β-surfaces of the molecule, than saturated phospholipids. Data from freeze-fracture electron microscopy show that cholesterol interacts preferentially with $[C_{16:0}-C_{18:1}]$ phosphatidylcholine in a 1:1 mixture of $[C_{16:0}-C_{18:1}]$ and $[C_{16:0}-C_{16:0}]$ phosphatidylcholine bilayers at 23 C (42). I believe that the difference in T_m is secondary for the preferential interaction, and the existence of $tt\Delta_{9,10}tg$ configuration in $[C_{16:0}-C_{18:1}]$ phosphatidylcholine to fit the β-surface of the steroid nucleus is a more direct interpretation of the freeze-dracture data.

Examination of Other Models

Many different molecular models for the cholesterol-egg phosphatidylcholine complexes in bilayer membranes have been proposed in recent years (38,46,49,50). With the exception of Bockerhoff (46), all other models were based on the contention that the hydroxyl group of the cholesterol forms a hydrogen bond to the phosphate group of phosphatidylcholine molecule. On the basis of our recent ^{31}P NMR experiments (11), it is clear that such a hydrogen

bond is undetectable. Moreover, if the cholesterol molecule is moved up to allow the C_2 and C_3 of the steroid nucleus to be in the hydrated region of the phosphate group, which is required to form such a hydrogen bond as suggested by Green et al. (49), then the other end of the steroid nucleus (C_{16} and C_{17}) will be positioned at approximately the same level as C_5 of the fatty acyl chains. Such a spatial arrangement will not allow the steroid nucleus to selectively affect the ^{13}C relaxation times of the carbons located in the center portion of the fatty acyl chain, including the $\Delta_{9,10}$ olefinic carbons, as indicated by the ^{13}C NMR data of Keough et al. (40) and Godici and Landsberger (41). Finally, if cholesterol were hydrogen bonded to the phosphate, then one would expect a *decrease* in hydration due to expulsion of water, when in fact a cholesterol-induced *increase* in hydration in the polar headgroup of egg phosphatidylcholine molecules is observed (43,45). The weight of evidence summarized above points clearly to the conclusion that hydrogen bonds between the sterol hydroxyl group and the phospholipid phosphate group must be present in very low amounts, if at all.

Based on energetic arguments, Brockerhoff (46) also concludes that there is no hydrogen bond between cholesterol hydroxyl group and phospholipid phosphate group. Brockerhoff's model differs from the present model, however, since it is concerned primarily with the relationship between hydrogen bond formation at the interfacial region of bilayers and the corresponding change in permeability properties of bilayers. No specific configuration of atomic arrangement in either the interfacial region or the hydrophobic region is given in Brockerhoff's model. In addition, a difference of detail is that the carbonyl oxygen of the position 2 ester linkage has beeen suggested by Brockerhoff to be a better H-bond acceptor for cholesterol, whereas in the present model, the H-bond between the –OH group of cholesterol and the carbonyl oxygen in position 2 is regarded as less common because the unsaturated 2-position is packed preferentially to the puckered β-surface of the steroid nucleus (see Figure 3 in reference 51).

ACKNOWLEDGMENT

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Comparative Effects of (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate on Acetyl CoA Carboxylase and Fatty Acid and Cholesterol Synthesis in vivo

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ABSTRACT

(—)-Hydroxycitrate and (+)-*allo*-hydroxycitrate were investigated for their effects on lipid synthesis in vivo under conditions of either high carbohydrate feeding or 24 hr fasting. Changes in rates of lipid synthesis resulting from the oral administration of these compounds were monitored with the use of radiolabeled H_2O, alanine, and acetate. In the fed rat, (—)-hydroxycitrate significantly reduced the incorporation of H_2O and alanine into fatty acids and cholesterol. An increased incorporation of labeled H_2O into fatty acids but no change in cholesterol synthesis in the fasted rat suggested that (—)-hydroxycitrate may be an activator of acetyl CoA carboxylase. With (—)-hydroxycitrate administration, acetate incorporation into fatty acids and cholesterol was subject to pool dilution effects under fed or fasted states. (+)-*allo*-Hydroxycitrate was ineffective in modulating the rates of fatty acid synthesis under either nutritional condition. Both (—)-hydroxycitrate and (+)-*allo*-hydroxycitrate were shown to be in vitro activators of acetyl CoA carboxylase, the former being a much stronger activator than the latter. Thus, stereospecificity of the hydroxycitrate isomers was demonstrated in both the inhibition of lipid synthesis (previously shown to occur at adenosine triphosphate citrate lyase) and the stimulation of fatty acid synthesis (possibly occurring at acetyl CoA carboxylase).

INTRODUCTION

(—)-Hydroxycitrate has been demonstrated to be a potent competitive inhibitor of adenosine triphosphate (ATP) citrate lyase (1,2). This enzyme is important in maintaining the acetyl CoA pool for fatty acid and cholesterol synthesis (3), particularly during the hyperlipogenic nutritional state produced by high carbohydrate feeding (4,5). This is supported by in vivo and in vitro studies demonstrating (—)-hydroxycitrate to be an effective inhibitor of fatty acid (6-13) and cholesterol (4,14,15) synthesis. The parallel response of fatty acid and cholesterol synthesis to (—)-hydroxycitrate suggests that a common mechanism, consisting of a shortage of cytoplasmic acetyl CoA, is responsible for both effects. The recent demonstration of acetoacetyl CoA thiolase and HMG CoA synthetase activity in hepatic cytosol (16-18) indicates that fatty acid and cholesterol synthesis share a common substrate pool of cytoplasmic acetyl CoA.

(—)-Hydroxycitrate was also shown to activate acetyl CoA carboxylase in vitro with potency equivalent to (19) or greater than (2) citrate. This was not surprising since various carobxylic acids were reported to activate this enzyme (20-22). If (—)-hydroxycitrate activates acetyl CoA carboxylase, then a stimulation rather than inhibition of lipid synthesis could result under metabolic conditions when ATP citrate lyase was not the major contributor to the acetyl CoA pool. For example, ATP citrate lyase apparently has limited importance in mouse liver (23) and adult (but not fetal) guinea pig liver (24).

The present studies were designed to evaluate the influence of (—)-hydroxycitrate on fatty acid and cholesterol synthesis in vivo, determined by the incorporation of $[^3H]_2O$, $[^{14}C]$ or $[^3H]$acetate and $[^{14}C]$alanine during different nutritional states: (a) high carbohydrate feeding, when ATP citrate lyase is a major contributor to the acetyl CoA pool (4,5) and citrate levels are elevated, thus supporting an activated acetyl CoA carboxylase; and (b) fasting, when the significance of ATP citrate lyase in maintaining the acetyl CoA pool is unknown, and when citrate levels are reported to be significantly depressed (25-27), the latter suggesting a reduced activation of acetyl CoA carboxylase. (+)-*allo*-Hydroxycitrate was employed because previous studied demonstrated it to be ineffective in altering hepatic rates of fatty acid synthesis in vivo (7). The capacity of both isomers, (—)-hydroxycitrate and (+)-*allo*-hydroxycitrate, to activate acetyl CoA carboxylase in vitro was also investigated.

MATERIALS AND METHODS

Animal Preparation and Diet

Female rats (Charles River Breeding Laboratories, Wilmington, MA) weighing 160-180 g were housed in wire-bottomed cages in a light-controlled (light 6 a.m. to 6 p.m., dark 6 p.m. to 6 a.m.), temperature-regulated (22 C) environment for a minimum of 1 wk prior to each experiment. During this period, they had free access to water and food (Purina Rodent Chow, Ralston Purina Co., St. Louis, MO). Following the 1 wk adjustment, animals were fasted 48 hr, then meal-fed for 6 to 9 days from 8 to 11 a.m., daily. The G-70 diet consisted of 70% glucose, 23% vitamin-free casein, 5% Phillips and Hart salt mixture IV, 1% corn oil, 1% vitamin mix, and 40 g/kg cellulose as reported previously (28).

In vivo Rates of Hepatic Lipogenesis and Cholesterogenesis

On the day of the experiment, rats were given orally by intubation 10 ml/kg of either physiological saline, (—)-hydroxycitrate trisodium salt (2.63 or 5.26 mmoles/kg), or (+)-*allo*-hydroxycitrate lactone (5.26 mmoles/kg) which was converted to the trisodium salt by saponification in NaOH, as reported previously (7). In the fed studies, rats were presented the 3 hr meal immediately after the oral intubation; in the fasted studies, rats were intubated 21 hr after the last meal. In all studies, the radiolabeled pulse was administered intravenously ½ hr prior to sacrifice and 3 hr after the oral administration. Three types of radiolabeled precursors were used: (a) 5 μCi [U-^{14}C] alanine, 12.3 mg alanine, 30.6 mg α-ketoglutaric acid and 1 mCi [^{3}H]$_2$O per animal per 0.25 ml saline; (b) 15 μCi [2-^{14}C]acetate, 15 ml acetate and 1 mCi [^{3}H]$_2$O per animal per 0.25 ml saline; or (c) 5 μCi [U-^{14}C]alanine, 12.3 mg alanine, 30.6 mg α-ketoglutaric acid, 15 μCi [^{3}H]acetate, and 15 mg acetate per animal per 0.25 ml saline. Lipogenesis and cholesterogenesis were determined as previously described (7,8,29). Briefly, livers were excised, weighed and saponified overnight in either 10% alcoholic KOH at 75 C, or 5 N NaOH at 90 C. Neutral lipids were extracted with petroleum ether, cholesterol was precipitated by the digitonin procedure (30), and radioactivity was determined in a liquid scintillation spectrometer. The saponified fatty acids were acidified with HCl extracted from the aqueous phase with petroleum ether and radioactivity was determined in a liquid scintillation spectrometer. Data are expressed as μmoles [^{3}H]$_2$O or [^{3}H]acetate and nmoles [^{14}C]acetate or [^{14}C]alanine converted to fatty acids or cholesterol per g liver per 30 min.

In vitro Activation of Acetyl CoA Carboxylase

Rats were meal fed a G-70 diet for 3 to 6 days as described above. Following the meal, livers were excised, homogenized in 3 volumes of 0.15 M KCl in 4 mM $MgCl_2$, and centrifuged at 105,000 g for 60 min. The supernatant (0.5 mg protein per assay) was preincubated at 37 C for 30 min in a volume of 0.7 ml containing 35 μmoles Tris-HCl pH 7.4, 14 μmoles $MgCl_2$, 0.7 μmoles glutathione, 0.63 mg bovine serum albumin, and the indicated concentration of either (—)-hydroxycitrate or (+)-*allo*-hydroxycitrate. After the activation of acetyl CoA carboxylase, the reaction was begun by addition of 2 mM ATP, 0.2 mM acetyl CoA, and 10 mM NaH[^{14}C]O$_3$ (10 μCi) in a final concentration of 1 ml (2). The reaction was stopped with 0.2 ml 6 N HCl after 10 min. The reaction mixture was evaporated at 50 C under nitrogen, resuspended in 0.25 ml H_2O, dissolved in a BBOT aqueous scintillation cocktail (8), and radioactivity determined in a liquid scintillation spectrometer.

Source of Chemicals

(—)-Hydroxycitrate (trisodium salt) and (+)-*allo*-hydroxycitric acid lactone were synthesized by Drs. Guthrie and Kierstead (Roche Research Division, Hoffmann-La Roche Inc., Nutley, NJ). Radiolabeled compounds were purchased from New England Nuclear, Boston, MA). Dietary components were obtained from Nutritional Biochemicals Corp. (Cleveland, OH). Acetyl CoA was purchased from P-L Biochemicals Inc. (Milwaukee, WI) and glutathione from Schwarz-Mann (Orangeburg, NY). Other chemicals were purchased from Sigma Chemicals (St. Louis, MO).

Statistical Analysis

A two-tailed "t" test was used to analyze results (31). All data were processed for outliers (32).

RESULTS

Effects of (—)-Hydroxycitrate on Lipogenesis and Cholesterogenesis in the Fed and Fasted Rat

The influence of (—)-hydroxycitrate, administered orally by intubation, on hepatic rates of fatty acid (Table I) and cholesterol synthesis (Table II) was examined by determining the incorporation of [^{3}H]$_2$O, [^{14}C] alanine, and [^{14}C] and [^{3}H]acetate into these lipids. [^{3}H]$_2$O was employed to determine the overall rate of fatty acid and cholesterol syn-

TABLE I

Influence of (—)-Hydroxycitrate on Fatty Acid Synthesis in the Fed and Fasted Rat

Treatment[a]	Precursor	Rate of lipogenesis[b]			
		Fed		Fasted	
		μmoles [^{3}H] converted	nmoles [^{14}C] converted	μmoles [^{3}H] converted	nmoles [^{14}C] converted[b]
Control	[^{3}H]$_2$O and [^{14}C]alanine	27.7 ± 4.2	697 ± 94	2.73 ± 0.40	31.1 ± 6.1
(—)-Hydroxycitrate	[^{3}H]$_2$O and [^{14}C]alanine	16.4 ± 1.5[c]	305 ± 54[d]	6.24 ± 0.47[d]	40.7 ± 5.2
Control	[^{3}H]$_2$O and [^{14}C]acetate	32.2 ± 2.7	831 ± 64	2.35 ± 0.30	66.9 ± 4.4
(—)-Hydroxycitrate	[^{3}H]$_2$O and [^{14}C]acetate	12.6 ± 2.4[d]	1399 ± 61[d]	4.78 ± 0.93[c]	124.5 ± 14.4[c]
Control	[^{3}H]acetate and [^{14}C]alanine	0.40 ± 0.02	644 ± 106	0.05 ± 0.01	41.1 ± 7.6
(—) Hydroxycitrate	[^{3}H]acetate and [^{14}C]alanine	1.11 ± 0.12[d]	220 ± 23[d]	0.12 ± 0.02[d]	32.2 ± 4.8

[a]Groups of 9 to 10 rats each were prefasted 48 hr, then meal-fed the G-70 diet for 6 to 9 days. The fed animals were then administered saline or (—)-hydroxycitrate (2.63 mmoles/kg) by stomach tube directly before the meal. The fasted animals were administered saline or (—)-hydroxycitrate (2.63 mmoles/kg) by stomach tube 21 hr after the last meal. Livers were assayed for in vivo rates of synthesis 3 hr later.

[b]*Data are expressed as μmoles [^{3}H]$_2$O, or μmoles [^{3}H]acetate, nmoles [^{14}C]alanine or nmoles [^{14}C]acetate converted per g liver per 30 min. Each value is the group mean ± SE.*

[c]$P < 0.05$.

[d]$P < 0.01$.

TABLE II

Influence of (—)-Hydroxycitrate on Cholesterogenesis in the Fed and Fasted Rat

Treatment[a]	Precursor	Rate of cholesterogenesis[a]			
		Fed		Fasted	
		μmoles [^{3}H] converted	nmoles [^{14}C] converted	μmoles [^{3}H] converted	nmoles [^{14}C] converted
Control	[^{3}H]$_2$O and [^{14}C]alanine	0.68 ± 0.09	15.7 ± 2.3	0.18 ± 0.03	0.67 ± 0.09
(—)-Hydroxycitrate	[^{3}H]$_2$O and [^{14}C]alanine	0.41 ± 0.07[c]	7.1 ± 0.7[d]	0.15 ± 0.02	0.76 ± 0.11
Control	[^{3}H]$_2$O and [^{14}C]acetate	1.35 ± 0.21	20.1 ± 3.1	0.24 ± 0.08	4.68 ± 0.97
(—)-Hydroxycitrate	[^{3}H]$_2$O and [^{14}C]acetate	0.75 ± 0.14[c]	34.6 ± 4.0[c]	0.25 ± 0.06	9.83 ± 1.46[c]
Control	[^{3}H]acetate and [^{14}C]alanine	0.03 ± 0.01	25.7 ± 3.1	0.007 ± 0.001	0.80 ± 0.07
(—)-Hydroxycitrate	[^{3}H]acetate and [^{14}C]alanine	0.05 ± 0.01	6.7 ± 0.9[d]	0.030 ± 0.009[c]	1.07 ± 0.25

[a]See footnote "a" in Table I for experimental protocol.

[b]Data are expressed as μmoles [^{3}H]$_2$O, or μmoles [^{3}H]acetate, nmoles [^{14}C]alanine or nmoles [^{14}C]acetate converted into cholesterol per g liver per 30 min. Each value is the group mean ± SE.

[c]$P < 0.05$.

[d]$P < 0.01$.

TABLE III

Comparative Effects of (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate on Fatty Acid Synthesis in the Fed and Fasted Rat

Treatment[a]	Rate of lipogenesis[b]	
	μmoles ^3H_2O converted	nmoles [^{14}C]acetate converted
Fed		
Control	38.1 ± 5.0	870 ± 54
(—)-Hydroxycitrate	22.3 ± 3.3[c]	1828 ± 140[d]
(+)-*allo*-Hydroxycitrate	32.8 ± 2.1	889 ± 46
Fasted		
Control	2.59 ± 0.25	45.3 ± 2.3
(—)-Hydroxycitrate	4.21 ± 0.70[c]	95.1 ± 13.3[c]
(+)-*allo*-Hydroxycitrate	2.87 ± 0.55	43.7 ± 4.8

[a]Six groups of 9 to 15 rats each were prefasted 48 hr, then meal-fed the G-70 diet for 6 to 8 days. They were then administered saline or 5.26 mmoles/kg of (—)-hydroxycitrate or (+)-*allo*-hydroxycitrate by stomach tube immediately before the meal, or 21 hr after the last meal in the fed and the fasted rat, respectively. Livers were assayed for in vivo rates of fatty acid synthesis 3 hr later. The radiolabeled precursor (consisting of 15 mg acetate, 15 μCi [^{14}C]acetate, and 1 mCi ^3H_2O) was administered intravenously 30 min before sacrifice.

[b]Data are expressed as μmoles ^3H_2O or nmoles [^{14}C]acetate converted into fatty acids per g liver per 30 min. Each value is the group mean ± SE.

[c]$P < 0.05$.

[d]$P < 0.01$.

thesis, since it was incorporated into these lipid classes independent of the source of carbon precursors of acetyl CoA (33-35). In the fed rat, when [^{3}H]$_2$O and [^{14}C]alanine were the precursors used, (—)-hydroxycitrate produced a 41% to 61% decrease in the overall rate of lipogenesis (as measured by [^{3}H]$_2$O), whereas a 56% to 66% inhibition of the incorporation of [^{14}C]alanine was observed. When [^{3}H]acetate or [^{14}C]acetate were the precursors employed, an increase in the apparent rate of synthesis, amounting to 278% and 168% of control, respectively, was demonstrated in the presence of (—)-hydroxycitrate. When (—)-hydroxycitrate was given to 21-hr fasted animals, different effects on the in vivo rates of fatty acid synthesis were observed, compared to the fed state. The incorporation of [^{14}C]alanine into fatty acids was not significantly changed in the fasted rat by treatment with (—)-hydroxycitrate. However, in the same system, the incorporation of [^{3}H]$_2$O indicated a twofold increase in the rate of synthesis. When [^{3}H]acetate or [^{14}C]acetate was the precursor used, (—)-hydroxycitrate treatment resulted in a 240% and 186% increase, respectively.

The effect of (—)-hydroxycitrate on cholesterol synthesis in the fed rat (Table II) was similar to that observed for fatty acid synthesis. When [^{3}H]$_2$O was the precursor used, a 40% to 54% decrease in synthesis was noted; a 55% to 74% decrease in the conversion of [^{14}C]alanine was also observed. If [^{3}H]acetate or [^{14}C]acetate was the precursor employed, the observed rates of cholesterol synthesis were 167% and 172% of control, respectively. As observed with fatty acid synthesis in the fasted rat, [^{14}C]alanine incorporation into cholesterol was unchanged, but the incorporation of [^{3}H]$_2$O into cholesterol was also unchanged, indicating that the overall rate of cholesterol synthesis was unaffected by treatment with (—)-hydroxycitrate. When cholesterogenesis was monitored by the conversion of [^{3}H]acetate or [^{14}C]acetate a 429% and 210% increase, respectively, in the rate was observed in the (—)-hydroxycitrate-treated groups.

Similar results were demonstrated in a comparison of intestinal and hepatic lipogenesis (data not shown). Following (—)-hydroxycitrate treatment in the fed rat, [^{14}C]alanine incorporated into intestinal fatty acids decreased (56% of control) while [^{3}H]acetate incorporation increased (171% of control). In the fasted rat, intestinal lipogenesis measured by [^{14}C]alanine remained unchanged, while the rates obtained with [^{3}H]acetate demonstrated a twofold increase.

Comparison of (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate on Lipogenesis in the Fed and Fasted Rat

In a comparison of (+)-*allo*-hydroxycitrate to (—)-hydroxycitrate in the fed rat model (Table III), the (+)-*allo*-hydroxycitrate did not inhibit the incorporation of [^{3}H]$_2$O (86% of control) nor increase the conversion of [^{14}C]acetate (102% of control) into fatty acids. The effect of (—)-hydroxycitrate in reducing the

conversion of $[^3H]_2O$ (59% of control) and in stimulating the incorporation of [^{14}C]acetate (210% of control) into fatty acids was similar to the earlier observations (Table I). In the fasted rat, (+)-*allo*-hydroxycitrate demonstrated no effect on lipogenesis from either $[^3H]_2O$ or [^{14}C]acetate. However, (—)-hydroxycitrate stimulated significantly the lipogenic rate from $[^3H]_2O$ (166% of control) and [^{14}C]acetate (210% of control). These effects were similar to those presented in Table I.

In vitro Activation of Acetyl CoA Carboxylase by (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate

The capacity of (—)-hydroxycitrate and (+)-*allo*-hydroxycitrate to activate acetyl CoA carboxylase in vitro was investigated. The concentration-dependent stimulation of acetyl CoA carboxylase by (—)-hydroxycitrate and (+)-*allo*-hydroxycitrate resulted in K_m values of 3 mM and 20 mM, respectively (Fig. 1). Previous reports indicate that citrate activated acetyl CoA carboxylase with a K_m of 1.9 mM (19) and 6.7 mM (2). Enzyme activity was maximal at 14.3 mM of both hydroxycitrate isomers and higher concentrations resulted in decreased activity (data not shown). Activation of acetyl CoA carboxylase by (—)-hydroxycitrate at 14.3 mM was twice as effective as a similar concentration of (+)-*allo*-hydroxycitrate; 0.7 mM (—)-hydroxycitrate was seven times as effective as an equivalent concentration of (+)-*allo*-hydroxycitrate.

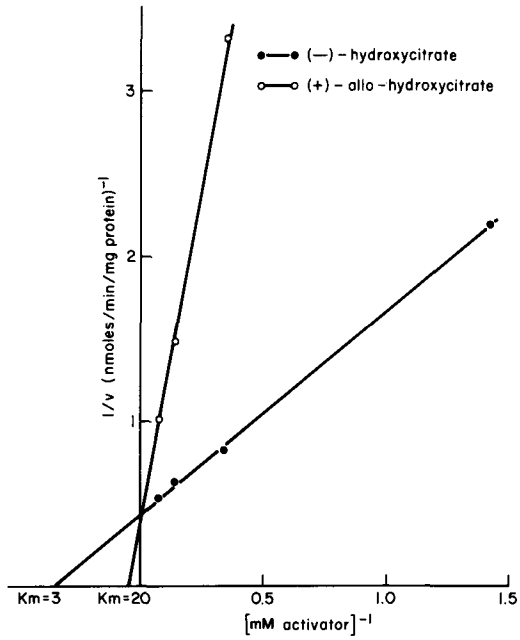

FIG. 1. Activation of Acetyl CoA Carboxylase by (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate. Hepatic high speed supernatant (105,000 x g) was preincubated for 30 min with the indicated concentrations of (—)-hydroxycitrate and (+)-*allo*-hydroxycitrate, in the absence of citrate.

DISCUSSION

Effects of (—)-Hydroxycitrate and (+)-*allo*-Hydroxycitrate on the Total Rate of Lipid Synthesis in the Fed and Fasted Rat

The total rate of lipid synthesis is determined by the incorporation of $[^3H]_2O$ into fatty acids and cholesterol. In the present investigation, the data obtained when $[^3H]_2O$ was the lipogenic precursor clearly indicate that in a high carbohydrate fed rat, the primary action of (—)-hydroxycitrate is to inhibit hepatic fatty acid and cholesterol synthesis, while (+)-*allo*-hydroxycitrate has no effect on fatty acid synthesis. These data confirm and extend previous findings (7,8) in which [^{14}C] alanine and/or $[^3H]_2O$ were used as precursors.

The increased incorporation of $[^3H]_2O$ label into fatty acids following (—)-hydroxycitrate treatment of the fasted rat, suggests stimulation at some site in the lipogenic pathway. The demonstration of the presence of cytosolic HMG CoA synthase and acetoacetyl CoA thiolase in avian and rat liver (16-18), and the inhibition of both fatty acid and cholesterol synthesis by (—)-hydroxycitrate in the fed rat, is evidence that cytosolic acetyl CoA is a precursor in both pathways. This would suggest that if (—)-hydroxycitrate were stimulating some step prior to acetyl CoA synthesis, then both fatty and cholesterol synthesis should be increased in the fasted rat. The unchanged rate of $[^3H]_2O$ label incorporation into cholesterol implies that (—)-hydroxycitrate acts at some point subsequent to acetyl CoA formation.

The possibility that (—)-hydroxycitrate (a tricarboxylic acid) may be activating the acetyl CoA carboxylase in vivo is supported by evidence that acetyl CoA carboxylase is activated by tricarboxylic acids (20-22) and particularly by citrate (36). In the fasted rat, the intracellular concentration of citrate was reported to fall from 0.262 to 0.151 μmoles/g wet liver wt following a 27 hr fast (25), and some reports indicated a 90% decrease in cytosol-localized citrate (26). Thus, during fasting, (—)-hydroxycitrate may replace citrate as an activator of acetyl CoA carboxylase. In the fed rat, citrate concentration is sufficient to fully activate the carboxylase, thus in this situation (—)-hydroxycitrate would be unnecessary as a tricarboxylic acid activator.

It was expected that (+)-*allo*-hydroxycitrate

would also activate acetyl CoA carboxylase in vivo since many tricarboxylic acids have been shown to function in this capacity (20-22). However, the unchanged rate of incorporation of $[^3H]_2O$ label into fatty acids in response to (+)-*allo*-hydroxycitrate by the fasted rat, implies that acetyl CoA carboxylase is not activated to any great extent by this isomer. The high K_m (20 mM) required for activation may indicate that the concentration reaching the liver is insufficient to activate acetyl CoA carboxylase.

Effect of Changes in the Acetyl CoA Pool

Both [^{14}C]alanine and [^{14}C]acetate were used to study the flux of carbon through the acetyl CoA pool. Alanine was used because its conversion to acetyl CoA can be regulated at the ATP citrate lyase level (7,8). Although acetate has been criticized as a precursor for cholesterogenesis (35,37-40), it was utilized because it can enter the acetyl CoA pool via the cytosolic acetyl CoA synthetase (41,42), an enzyme not known to be affected by (—)-hydroxycitrate.

In the fed rat, the inhibitory action of (—)-hydroxycitrate on ATP citrate lyase is expressed in a reduction of the cytosolic acetyl CoA concentration as shown by the decreased flow of $[^3H]_2O$ and [^{14}C]alanine label into fatty acids and cholesterol. As a result of the (—)-hydroxycitrate-induced decrease in the endogenous acetyl CoA pool, [^{14}C]acetate is less diluted and an increased flow of [^{14}C] acetate label into both fatty acids and cholesterol occurred.

In the fasted rat, lipid synthesis is reduced as is the activity of the lipogenic enzymes. Gluconeogenesis from amino acids is increased (43), thus the contribution of alanine to the acetyl CoA pool may be decreased. The increase in fatty acid synthesis from $[^3H]_2O$ following (—)-hydroxycitrate treatment is not mirrored by increased [^{14}C]alanine incorporation into fatty acids. This may be the result of thy simultaneous activation of acetyl CoA carboxylase and inhibition of ATP citrate lyase by (—)-hydroxycitrate, as well as the possibility that the lyase may not be as important in producing acetyl CoA for fatty acid synthesis during fasting. The increased incorporation of [^{14}C]acetate into fatty acids may be the result of the activation of acetyl CoA carboxylase. The simultaneous elevation of [^{14}C]acetate and the unaltered rate of $[^3H]_2O$ incorporation into cholesterol, suggests a pool dilution phenomenon, resulting from the increased demand for acetyl CoA carbon for fatty acid synthesis.

A previous study has also described the effect of (—)-hydroxycitrate on the incorporation of $[^3H]_2O$ and [^{14}C]acetate into fatty acids and cholesterol, in perfused liver and liver homogenates (14,44). Reported changes in the rates of fatty acid synthesis were similar to those we observed in vivo in the fasted rat; the alterations reported in the rate of cholesterol synthesis corresponded to neither our fed or fasted in vivo rates of cholesterogenesis. This emphasized the problems that may be encountered in studying metabolite flux in in vitro compared to in vivo systems.

The (—)-hydroxycitrate-induced stimulation of fatty acid synthesis in the fasted rat suggests that, in metabolic conditions where acetate is the primary contributor to the acetyl CoA pool, such as in fed ruminants (45), fasted rats (46), or ethanol-treated mammals, (—)-hydroxycitrate may stimulate rather than inhibit fatty acid synthesis.

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Determination of the Absolute Configuration at C-20 and C-24 of Ergosterol in Ascomycetes and Basidiomycetes by Proton Magnetic Resonance Spectroscopy

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ABSTRACT

Samples of ergosterol isolated from *Saccharomyces cerevisiae, Neurospora crassa,* and *Agaricus sp.*, and commercial ergosterol all displayed identical proton magnetic resonance (PMR) spectra at 220 MHz. From the effects produced on the doublet for C-21 by epimerization at C-20 and C-24 in sterols of known configuration, the absolute configurations at these positions in ergosterol were determined. The data demonstrate that ergosterol from both Ascomycetes and Basidiomycetes is the same and that at C-20 and C-24, the two H-atoms are on the α-side of the asymmetric carbon atoms and that C-22 is *trans*-oriented with respect to C-13 about the 17(20)-bond.

INTRODUCTION

Ergosterol is believed to be a common constituent of Ascomycetes and Basidiomycetes (1). The configurations at C-20 and C-24 were examined early in the history of steroids (2) and the one at C-24 was studied still more recently by Tsuda et al. (3). The latter authors were able to show conclusively that in stigmasterol the 24-ethyl group is α-oriented reversing the assignment made by Bergmann and Low (4). Bergmann and Low (4) also reported stigmasterol and ergosterol to have the same configuration, so Tsuda et al. (3) proceeded to define the configuration in ergosterol. Unfortunately, they were unable to bring the work to the same level of security as they had done with stigmasterol. While their evidence pointed strongly to a methyl group on the β-side of C-24, questions remained, and they were laudably cautious in claiming to have proved the configuration.

Recently using proton magnetic resonance (PMR) spectroscopy at 220 MHz, we (5) and Mulheirn (6) demonstrated unequivocally that in a given plant both 24α- and 24β-methylsterols can, and in Tracheophytes frequently (5) do coexist contrary to previous beliefs. We also presented evidence for the existence of a mixture of ergosterol and 24-epiergosterol in *Lycopodium complanatum* (7). The possible existence of 24-epiergosterol coupled with the uncertainty left from the work of Tsuda et al. (3) and, the fact that they do not report the biological origin (generally presumed to be yeast) of the ergosterol they used, persuaded us to reexamine the problem using the PMR technique. At the same time, it became possible to use PMR to reexamine the configuration assigned at C-20 which, as that at C-24, depended on molecular rotation and m.p. data leaving something to be desired. We have been able to bring unequivocal confirmation to the previous configurational assignments at C-20 and C-24 of yeast ergosterol, and we have extended the analysis to the dominant sterol of another Ascomycete (*Neurospora crassa*) as well as to a Basidiomycete (*Agaricus sp.*). The latter two organisms also contained ergosterol rather than one of the three other possible epimers. In all cases, the PMR spectra indicated configurational purity unlike the situation in *Lycopodium complanatum* (7). In view of this, we suggest that "ergosterol" should be used only to connote the epimer with 20α- and 24α-H-atoms, and the terms 24-epi-, 20-epi- and 20,24-bisepiergosterol should be used for the epimers. The two 20-episterols, as examined in the Discussion section, probably do not exist naturally.

MATERIALS AND METHODS

Yeast, *Saccharomyces cerevisiae* (ATCC #18790) was cultured under aerobic conditions in a synthetically compounded yeast nitrogen base described by Andreasen and Stier (8) which was modified by the inclusion of only the following vitamins: biotin, calcium pantothenate, nicotinamide, pyridoxine-HCL and thiamine-HCL. *Neurospora crassa* (ATCC #9279) was cultured in a synthetically compounded medium described by Coursen and Sisler (9) which was modified by doubling the amount of KH_2PO_4 and K_2HPO_4 (H.D. Sisler, personal communication). A commercial mushroom represented the Basidiomycetes. It was identified by its morphological characteristics as belonging to the genus *Agaricus* but the precise species was uncertain. From *N. crassa*

was obtained 31 mg of crude sterol per 100 g of wet weight. From the *Agaricus sp.* 18 mg was obtained, and from *S. cerevisiae* 13 mg.

Sterols were isolated from the mixed free and esterified pools and examined by procedures previously described (for a key, see 5 and 7). The samples of ergosterol (finally purified by argentation chromatography and crystallized from methanol) each possessed the same rates of movement in a gas liquid chromatographic system (XE-60) and the same mass and PMR spectra at 220 MHz.

RESULTS AND DISCUSSION

The PMR spectra of all samples of ergosterol were superimposable on one another. The values for the various methyl groups are given in Table I together with those of authentic 24-epiergosterol. The latter was prepared by incubation of 24α-methylcholesterol with *Tetrahymena pyriformis* (7). It will be seen from Table I that the signal from C-21 is shifted downfield (2 Hz or 0.01 ppm) in the case of fungal ergosterol. The influence of configuration on the direction of the shift can be determined from the spectra of stigmasterol, for which the configuration is certain (3), and its epimer, poriferasterol. The signal for C-21 is downfield in the latter compared to the former (5,10). The stigmasterol-poriferasterol (24-ethyl-$\Delta^{5,22}$) pair differ from the ergosterol case (24-methyl-$\Delta^{5,7,22}$) in the unsaturation in ring B and in the size of the alkyl group at C-24. That neither these differences nor the presence of the Δ^{22}-bond have an influence on the direction of the shift is demonstrated by the spectra of the epimeric 24-methylcholesterols (24-methyl-Δ^{5}). The epimer (dihydrobrassicasterol) derived synthetically (11) from ergosterol displays the downfield shift compared to the other epimer (campesterol derived from soybeans) (5). Consequently, the shift in the signal from C-21 becomes an unequivocal method for determination of the absolute configuration at C-24. The epimer with the larger chemical shift has the β-oriented alkyl group. The ergosterol of the Ascomycete and Basidiomycete species examined must therefore possess a 24β-methyl group.

The position of the PMR signal for C-21 also can be used to determine the absolute configuration at C-20. This has become possible from recent work (12) in this laboratory in which we prepared 20-epicholesterol (also known as 20-isocholesterol). The 20-epicholesterol produced a signal for C-21 which was displaced upfield compared to cholesterol. The shift (0.10 ppm) is very much stronger than the one produced by

TABLE I

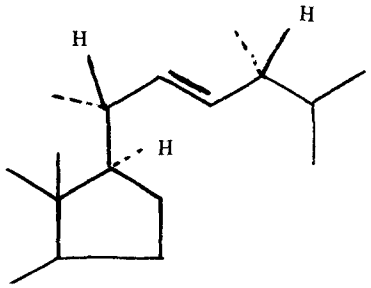
Proton Magnetic Resonance (PMR) Spectra of Ergosterol and 24-Epiergosterol at 220 MHz

Proton position	Chemical shift in ppm from trimethylsilyl (TMS) in $CDCl_3$	
	Ergosterol[a]	24-Epiergosterol[b]
C-18 (s)	0.63	0.63
C-29 (s)	0.95	0.95
C-21 (d)	1.04	1.03
J = 6 Hz		
C-26,27 (d)	0.84	0.84
J = 6 Hz	0.82	0.82
C-28 (d)	0.92	0.92
J = 6 Hz		

[a]From fungi
[b]See Ref. 7

epimerization at C-24, and, on this basis, the two effects are readily distinguishable. In the spectrum which we have already reported (5) of 24β-methylcholesterol derived synthetically from ergosterol, the signal from C-21 appears at 0.91 ppm. The value for animal cholesterol is 0.91 and for 20-epicholesterol is 0.81 (12). Since the effect of substitution at C-24 is of the order of 0.01-0.02 ppm (5,10), the similarity in the values for 24β-methylcholesterol and for animal cholesterol for which the configuration is known (2) mean that ergosterol must have the same configuration (20α-H-atom) as cholesterol. It is perhaps worth noting that the signal for C-21 from ergosterol and 24-epiergosterol (Table I) is displaced downfield from that in cholesterol and 24β-methylcholesterol due to the alkylic effect of the Δ^{22}-bond.

We have also recently demonstrated (12,13) that in cholesterol the preferred conformation of the 17(20)-bond is such as to produce a "right-handed" conformer, i.e. C-22 and the remainder of the side chain lie to the right when the nucleus is viewed in the usual way. In this conformation, C-21 and C-22 lie pseudo-equatorially on the back-side. This gives a skew conformation about the 17(20)-bond which accounts for the preference (12,13). By contrast, 20-epicholesterol possesses not only a β-oriented H-atom but, in order to maintain a skew conformation, assumes a "left-handed" conformation about the 17(20)-bond thereby placing C-21 in a different environment and producing a different PMR signal (12,13). The ability to deduce the configuration at C-20 is therefore integrally associated with the conformational question. The upfield shift in the PMR signal on passing from a 20α-H- to a 20β-H-atom is actually produced as a result of a change from a "right-handed" to a "left-handed" conformation which is a secondary,



Scheme 1. Stereochemistry of Ergosterol (Interrupted lines at C-20 and C-24 imply the subsitutent is behind the plane containing C-20, C-22, C-23, C-24, C-25, and C-27 or C-27 when arranged in the staggered conformation. Solid lines for substituents at C-20 and C-24 imply they project in front of this plane).

energy-minimizing effect of changing the configuration. The PMR spectrum of 24β-methylcholesterol derived from ergosterol proves, therefore, that the latter has the "right-handed" conformation (Scheme 1).

If, as generally believed in the cyclization leading to lanosterol, the bond-forming and bond-breaking reactions always occur in a *trans*-fashion, and if we further assume, as proposed elsewhere in detail (12), that appropriate skew conformations will arise at various stages of the cyclization of squalene oxide, it becomes necessary a priori for the end-product of cyclization to be a sterol with a 20α-H-atom and a "right-handed" side chain. Consequently, the two epimers of ergosterol which are empirically possible with a 20β-H-atom probably do not exist biologically. No such theoretical restriction applies at C-24, and 24-epiergosterol could reasonably and probably does (7) exist, although apparently not in fungi. Since there are compelling reasons for believing that the asymmetric center at C-24 is created in fungi by reduction of the $\Delta^{24(28)}$-bond of a 24-methylenesterol, the 24β-methyl configuration must have its origin in the side (α) of C-24 which is attacked by an H-atom in this reduction.

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Relative Utilization of Fatty Acids for Synthesis of Ketone Bodies and Complex Lipids in the Liver of Developing Rats

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ABSTRACT

The regulation of hepatic ketogenesis, as related to the metabolism of fatty acids through oxidative and synthetic pathways, was studied in developing rats. [1-^{14}C]palmitate was used as a substrate to determine the proportions of free fatty acids utilized for the production of ketone bodies, CO_2 and complex lipids. Similar developmental patterns of hepatic ketogenesis were obtained by measuring the production of either [^{14}C]acetoacetate from exogenous [1-^{14}C]palmitate or the sum of unlabeled acetoacetate and β-hydroxybutyrate from endogenous fatty acids. The production of total ketone bodies was low during the late fetal stage and at birth, but increased rapidly to a maximum value within 24 hr after birth. The maximal ketogenic capacity appeared to be maintained for the first 10 days of life. $^{14}CO_2$ production from [1-^{14}C]palmitate increased by two- to fourfold during the suckling period, from its initial low rate seen at birth. The capacity for synthesis of total complex lipids was low at birth and had increased by day 3 to a maximal value, which was comparable to that of adult fed rats. The high lipogenic capacity lasted throughout the remaining suckling period. When ketogenesis was inhibited by 4-pentenoic acid, the rate of synthesis of complex lipids did not increase despite an increase in unutilized fatty acids. During the mid-suckling period, approximately equal amounts of [1-^{14}C]palmitate were utilized for the synthesis of ketone plus CO_2 and for complex lipid synthesis. By contrast, in adult fed rats, the incorporation of fatty acids into complex lipids was four times higher than that of ketone plus CO_2. These observations suggest that stimulated hepatic ketogenesis in suckling rats results from the rapid oxidation of fatty acids and consequent increased production of acetyl CoA, but not from impaired capacity for synthesis of complex lipids.

INTRODUCTION

In an earlier study, we showed that hyperketonemia of developing rats began after the first postnatal feeding and persisted throughout the suckling period (1). The elevated level of ketone bodies (KB) was accompanied by high levels of free fatty acids, a low level of insulin, and a glucose level comparable to that of adult rats, as similarly reported by others (2,3). Plasma concentrations of insulin and KB were reciprocally related, as were plasma insulin and hepatic ketogenesis in suckling rats of various ages. Further, we found a close relationship between plasma levels of KB and ketogenic capacities of the liver implicating an increased rate of hepatic ketone synthesis as a cause of the ketosis of newborns. However, the precise mechanism(s) stimulating ketogenesis during postnatal development was not clear from these earlier studies.

Most information concerning the regulation of ketone metabolism has come from studies of adult laboratory animals in various nutritional and pathophysiological states resulting from starvation, fat-feeding, and diabetes (4-6). Such studies have clearly indicated an association between ketosis and alterations in lipid and carbohydrate metabolism. In suckling rats, such alterations may exist because newborn rats obtain energy primarily from the high fat content of milk (7). Free fatty acids can be either esterified in the liver to triglycerides, phospholipids and other lipids, or oxidized through β-oxidation (5,8) to produce energy and acetyl coenzyme A (AcCoA). AcCoA is an immediate precursor for the synthesis of KB and fatty acids, and it is a substrate for the further production of energy in the Krebs' Cycle. Therefore, the rate of ketogenesis might be regulated by the degree of competition for free fatty acids between β-oxidation and complex lipid synthesis (5,8). In the present studies, we related the amounts of free fatty acids claimed by each of these processes to the stimulated ketogenesis in rat liver during postnatal development. The results indicate that the increased utilization of fatty acids for ketogenesis during the suckling period is not associated with any impairment in hepatic

capacity for synthesis of complex lipids.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were used throughout. Female rats were bred, and a positive sperm test was used to estimate gestational age. Pregnant rats were kept in individual cages; the floor of each cage was covered by a layer of bedding (Ad-Sorb-Dri), an inch thick. Commercial Purina rat chow and water were always available. Fetuses of 20 and 22 days of gestation were obtained by caesarean section and were not fed before experiments. All pups used for studies during the lactation period were born naturally and were suckled by their dams until weaning on the twenty-second day. Adult male rats either fed ad libitum or fasted for 48 hr prior to the study were included for comparison.

Liver Homogenates

Immediately after the rats were decapitated, livers were removed and placed in ice-cold 0.9% NaCl. Livers weighing 1.0 to 1.2 g were homogenized in Ca^{2+}-free Krebs-Ringer phosphate buffer (pH 7.4) to obtain 20% (w/v) crude homogenates. Because of their small size, two to four livers were pooled from developing rats.

For fatty acid metabolism, 0.8 ml of the liver homogenate was added to 1.2 ml of the same buffer containing a final concentration of 1.5% bovine serum albumin (Fraction V). Each mixture was incubated in a metabolic shaker (90 strokes/min) at 37 C in the following studies.

Ketogenesis

To determine the hepatic capacity for ketone production, the liver homogenate containing endogenous fatty acids was incubated with or without exogenous unlabeled palmitate for 10 min. The supernatant of the incubated mixture was then prepared and assayed for KB as described previously (1). Acetoacetate (AcAc) and β-hydroxybutyrate (β-OHB) were determined enzymatically (9). The net rate of synthesis, expressed as μmole ketone formed/g liver/10 min, reflects the difference in amounts of KB obtained before and after incubation.

Fatty Acid Partition

To determine the relative rate of utilization of fatty acids through various metabolic pathways, liver homogenate was added to a 25 ml reaction flask containing [1-^{14}C]palmitate (280,000 dpm) and unlabeled palmitate (0.4 μmole) in a final volume of 2 ml. The rate of utilization was expressed as μmole of [1-^{14}C]palmitate incorporated into product/g liver/30 min.

Immediately after the addition of homogenate, the flask was flushed with an O_2-CO_2 mixture (95:5), and capped with a rubber stopper subtending a center well. The well contained a folded filter paper (2 x 2 cm) saturated with 0.2 ml 1 M Hyamine hydroxide. Thirty minutes after incubation, the reaction was terminated by adding 0.4 ml of 62% citric acid (w/v). $^{14}CO_2$ produced from oxidation of [1-^{14}C]palmitate was trapped in the filter paper. After the well and filter paper were removed, each flask was recapped with a rubber stopper that held another center well containing a filter paper. The rate of synthesis of [^{14}C]AcAc was essentially determined by the method described elsewhere (10). A 0.4 ml of a mixture of aniline and 62% citrate (1:1.5, v/v) was introduced to the reaction mixture to decarboxylate [^{14}C]AcAc. The released $^{14}CO_2$ was collected in the same manner as above and was used to calculate the amount of [^{14}C]palmitate incorporated into AcAc. The total ^{14}C from palmitate incorporated into AcAc was estimated by doubling the radioactivity counted because of even distribution of the label in carbon positions 1 and 3 of AcAc (11).

To measure the rate of synthesis of complex lipids from [1-^{14}C]palmitate, the homogenate was incubated as described above for the synthesis of [^{14}C]AcAc. At the end of a 30-min incubation, 0.5 ml of 2 N H_2SO_4 was added to the flask. A 1.0 ml aliquot was then transferred to a tube containing a 20 ml solution of chloroform and methanol (2:1, v/v). Lipids were extracted according to the procedure of Folch et al. (12). The final extract, resuspended in 0.2 ml of petroleum ether (b.p. 30-60 C), was spotted on a thin layer chromatograph plate (20 x 20 cm) coated with Silica Gel G, 0.5 mm thick. The plate was developed in a solvent system of hexane, diethyl ether, and glacial acetate (80:20:1, v/v/v). The plates were sprayed with 2,7-dichlorofluorescein, and placed under UV light; lipid classes were identified by comparison with standards. Each band corresponding to a different lipid fraction was scraped into a scintillation vial and dissolved in toluene scintillation fluid (4 gm Omnifluor + 230 ml ethanol + 770 ml toluene). Radioactivity of these fractions was determined by a liquid scintillation counter (Model Mark III, Searle Analytic, Inc.).

Chemicals

[1-^{14}C]palmitate and Omnifluor (a mixture

of 98% PPO and 2% p-Bis-(O-methyl)-benzene) were obtained from New England Nuclear, Boston, MA. β-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) was purchased from Boehringer and Mannehim Biochemicals, New York, NY. 2,7-Dichlorofluorescein and all lipid standards for thin layer chromatography were supplied by Applied Science Lab., Inc., State College, PA. Bovine serum albumin (Fraction V) and other chemicals were obtained from Sigma Chemicals Co., St. Louis, MO.

RESULTS

β-OHB and AcAc are the major KB present in the liver and peripheral blood. To determine which of the two was predominantly synthesized under in vitro systems used for the study of fatty acid metabolism, the hepatic capacity for synthesis of AcAc and β-OHB was measured in liver homogenates of developing rats. The net production of AcAc and β-OHB were taken together as a measure of total synthetic capacity. The production of total KB was low two days before term and remained low at birth, but the total capacity accelerated rapidly to a maximum within the first postnatal day (Fig. 1). This rate of synthesis was maintained over the next 10 days and then decreased sharply. Although AcAc production during the suckling period was always greater than total synthesis, both showed the same developmental pattern of change. The higher values of AcAc than the total of AcAc and β-OHB were a result of conversion of endogenous β-OHB to AcAc during incubation. The conversion of β-OHB to AcAc may possibly be explained by a shift in mitochondrial redox state toward the oxidized side (13).

In the liver of 10-day-old pups, the addition of palmitate at 0.05 mM and 0.1 mM increased total KB synthesis by 1.7- and 2.2-fold, respectively, with a concomitant decrease in the conversion of β-OHB to AcAc (Table I). Further increase of palmitate from 0.1 mM to 0.2 mM decreased AcAc synthesis, and resulted in a net production of β-OHB, but did not alter overall KB production. At higher concentrations, i.e., 0.4 mM and 0.6 mM, palmitate was found to be inhibitory to ketogenesis. Similarly, in 5-day-old pups, the synthesis of total KB increased with palmitate concentrations approaching 0.2 mM and decreased with concentrations exceeding this amount. When liver homogenates prepared from newborn rats at birth were incubated, exogenous palmitate at 0.2 mM and 0.6 mM did not stimulate synthesis of total KB or AcAc, although it appeared to maintain a net synthesis of β-OHB.

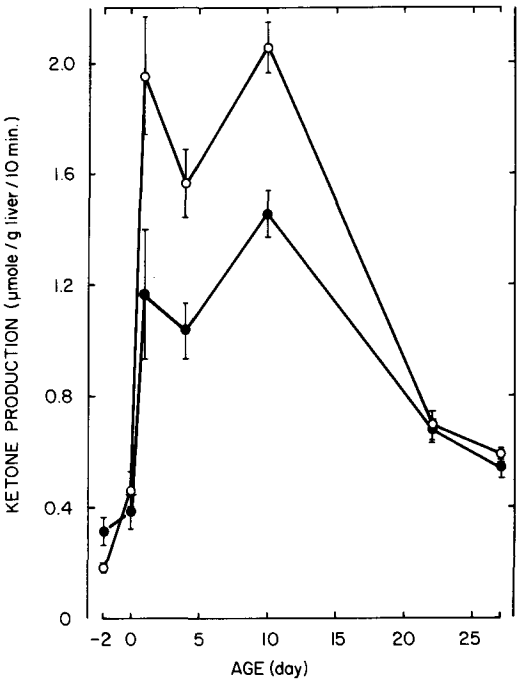

FIG. 1. Hepatic synthesis of ketone bodies from endogenous fatty acids in developing rats. Ketone production represents a net synthesis of acetoacetate (○—○) or sum of acetoacetate plus β-hydroxybutyrate (●—●) during 10 min incubation. Values are means ± SEM for eight samples of two to four pups each. 0 and -2 indicated in the abscissa represents newborn pups at birth and fetuses obtained 2 days before term, respectively.

The rate of fatty acid utilization for ketone synthesis was estimated by the production of [^{14}C] AcAc, because as shown in preceding experiments (Table I), AcAc was the major product in the presence of 0.2 mM palmitate, while β-OHB contributed only 9-14% of total KB synthesized. The incorporation of [1-^{14}C]palmitate into labeled AcAc was low prior to and immediately after birth and increased rapidly after birth, reaching a peak level within 10 to 15 days of life. Thereafter, the rate decreased sharply to that of adult fed rats. A high rate of AcAc synthesis from [1-^{14}C]palmitate was also observed in adult rats after deprivation of food for 48 hr. It is important to point out that the temporal changes in [^{14}C] AcAc synthesis during the suckling period follows the developmental changes in hepatic ketogenic capacities (Figs. 1 and 2).

Oxidation of [1-^{14}C]palmitate via Krebs' cycle was determined by measuring CO_2 production. The rate of oxidation was as low in fetuses and newborns as in adult rats, but

TABLE I

Effect of Exogenous Palmitate on Hepatic Ketogenesis in Developing Rats

Palmitate (mM)	Ketone production[a] (μmole/g liver/10 min)		
	AcAc	β-OHB	Total KB
		At birth	
0	0.46 ± 0.08[b]	-0.06 ± 0.04	0.40 ± 0.06
0.2	0.34 ± 0.06	0.14 ± 0.02	0.47 ± 0.07
0.6	0.33 ± 0.11	0.06 ± 0.06	0.40 ± 0.14
		5-day-old	
0	1.48 ± 0.16	-0.24 ± 0.01	1.23 ± 0.15
0.1	2.58 ± 0.20	-0.02 ± 0.05	2.56 ± 0.20
0.2	2.51 ± 0.04	0.26 ± 0.09	2.78 ± 0.12
0.4	0.82 ± 0.06	-0.04 ± 0.03	0.78 ± 0.07
0.6	0.69 ± 0.10	-0.10 ± 0.02	0.54 ± 0.12
0.8	0.36 ± 0.05	-0.01 ± 0.03	0.37 ± 0.06
		10-day-old	
0	2.26 ± 0.10	-0.60 ± 0.01	1.67 ± 0.09
0.05	3.19 ± 0.12	-0.35 ± 0.03	2.84 ± 0.15
0.1	3.64 ± 0.12	-0.05 ± 0.01	3.59 ± 0.08
0.2	3.05 ± 0.15	0.51 ± 0.14	3.56 ± 0.26
0.4	0.94 ± 0.08	-0.26 ± 0.04	0.67 ± 0.08
0.6	0.87 ± 0.02	-0.18 ± 0.02	0.69 ± 0.04

[a]Ketone production represents a net change in ketone bodies before and after 10 min incubation. Abbreviations: AcAc, acetoacetate; β-OHB, β-hydroxybutyrate; Total KB, sum of AcAc and β-OHB.

[b]Values are means ± SEM for four samples. Each sample consisted of livers from two to four pups depending on size. Minus values reflect negative synthesis resulting from conversion of β-OHB to AcAc during incubation.

increased significantly by two- to fourfold throughout the suckling period. The increased production of CO_2 suggests that more fatty acids derived from milk are oxidized for energy during the postnatal development.

To determine the capacity for synthesis of complex lipids from fatty acids, we measured the incorporation of [1-^{14}C]palmitate into phospholipids (PL), triglycerides (TG), diglycerides (DG), monoglycerides (MG), cholesterol (C), and cholesteroyl esters (CE). The rate of incorporation of [1-^{14}C]palmitate into total lipids was 0.63 ± 0.03 μmole/g liver/30 min two days before term, but had decreased by 50% at birth (Fig. 3). Within the next two days, however, the rate had surpassed that of the fetal period. On the third day, the rate of synthesis reached that of adult rats and stabilized at that level for the remaining suckling period. In adult rats, the synthesis was suppressed by about 50% after 48 hr of starvation. In other studies in vitro, lipid synthesis was not affected by increasing concentrations of exogenous fatty acids in developing rats (data not shown).

The distribution of [1-^{14}C]palmitate into individual lipid classes was determined by thin layer chromatography. This enabled us to estimate the contribution of each lipid to the total complex lipids synthesized. The results were expressed as percent of radioactivity distributed to individual lipid fractions (Table II). At birth, 78% of the total radioactivity incorporated into total complex lipids was found in PL + MG, 11% each was in DG + C and TG, while only 1% was found in CE. A similar distribution was demonstrated in the fetal livers. Using another solvent system of chloroform, acetone, and glacial acetate (88:12:0.05, v/v/v) to separate lipids, we observed that PL accounted for more than 95% of the total activity seen in PL + MG. In one-day-old rats, after the first feeding, the incorporation of [1-^{14}C]palmitate had shifted from PL + MG to TG, and DG + C, and the incorporation into CE remained unchanged. Thereafter, the proportion of [1-^{14}C]palmitate in PL + MG returned to the initial level found at birth or increased to a certain degree primarily at the expense of synthesis of DG + C and TG. The percent of TG incorporation in newborn and suckling rats was 1.6- to 2.4-fold the values of adult rats. The higher percentage of radioactivity associated with TG resulted mainly from a proportional decrease in PL synthesis. Thus, in suckling rats, the relative distribution of [1-^{14}C]palmitate in lipids was: PL + MG >

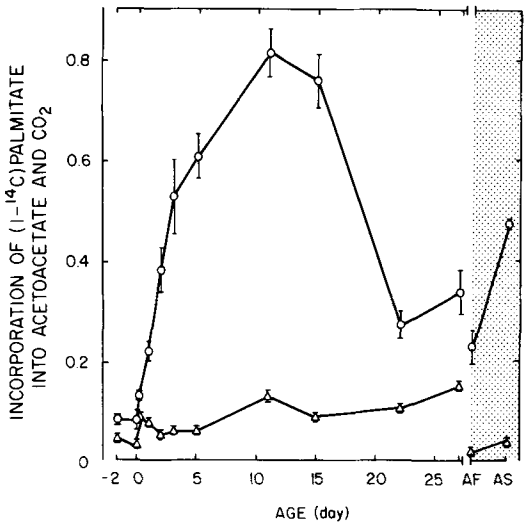

FIG. 2. Hepatic synthesis of acetoacetate and CO_2 from [1-^{14}C] palmitate in developing rats. The liver homogenates were incubated for 30 min in a final 2 ml of a medium containing [1-^{14}C] palmitate (280,000 dpm and 0.4 μmole). The rate of incorporation into [^{14}C] acetoacetate (○—○) and $^{14}CO_2$ (△—△) was expressed as μmole [1-^{14}C] palmitate incorporated/g liver/30 min. Values are means ± SEM for four to eight samples. Each sample consisted of two to four livers obtained from developing rats, whereas in adult fed (AF) or starved (AS) rats each sample was prepared from a single liver.

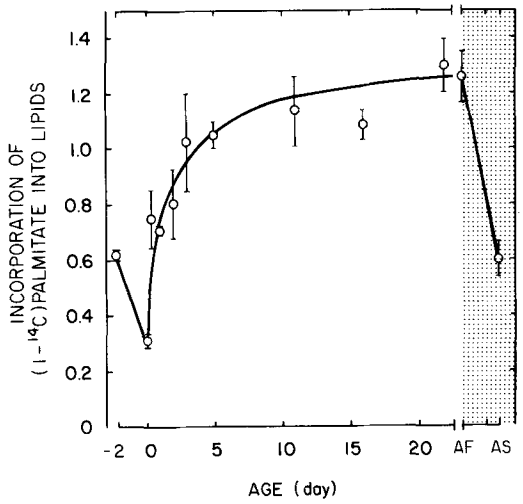

FIG. 3. Hepatic synthesis of complex lipids from [1-^{14}C] palmitate in developing rats. Incubation system was the same as described in Figure 2. The rate of incorporation into [^{14}C] complex lipids expressed as μmole [1-^{14}C] palmitate incorporated/g liver/30 min represents total synthesis of phospholipids, monoglycerides, diglycerides, triglycerides, cholesterol and cholesteroyl esters. Values are means ± SEM for four to eight samples. Each sample consisted of two to four livers obtained from developing rats, whereas in adult fed (AF) or starved (AS) rats each sample was prepared from a single liver.

TG > DG + C > CE.

To estimate the quantities of fatty acids claimed by both synthetic and oxidative pathways, we determined the percentage of [1-^{14}C] palmitate radioactivity distributed to complex lipids, AcAc, and CO_2 (Table III). The pattern of [1-^{14}C] palmitate partition into lipids and AcAc was similar in fetuses, newborns at birth, and adult fed rats. Consistent with the increased ketogenic capacity in suckling rats, the percent radioactivity in AcAc increased between 1 and 15 days of age and was

TABLE II

Percent Distribution of [1-^{14}C] palmitate in Various Lipids Synthesized in the Liver of Developing Rats

Age (day)	Lipid classes[a]			
	PL + MG	DG + C	TG	CE
20-day fetus	73.1 ± 2.6[b]	11.2 ± 1.2	13.4 ± 1.4	1.3 ± 0.4
At birth	77.5 ± 0.6	10.8 ± 0.5	10.6 ± 0.2	1.1 ± 0.1
1	69.8 ± 0.1	15.6 ± 0.4	13.2 ± 0.7	1.3 ± 0.6
2	75.6 ± 1.4	8.4 ± 1.2	14.2 ± 2.4	1.8 ± 0.1
3	76.5 ± 1.6	8.0 ± 1.4	13.9 ± 0.6	1.6 ± 0.4
5	79.4 ± 0.4	7.6 ± 0.3	11.7 ± 0.4	1.4 ± 0.2
11	84.7 ± 0.6	4.6 ± 0.1	9.4 ± 0.6	1.3 ± 0.1
15	83.7 ± 0.4	5.0 ± 0.4	9.6 ± 0.4	1.7 ± 0.1
27	83.6 ± 1.6	5.4 ± 0.6	8.4 ± 1.0	2.1 ± 0.3
Adult fed	86.8 ± 1.4	6.1 ± 1.4	5.8 ± 1.3	1.2 ± 0.1

[a]Lipid classes including phospholipids (PL), monoglycerides (MG), diglycerides (DG), cholesterol (C), triglycerides (TG) and cholesteroyl esters (CE) synthesized from [1-^{14}C] palmitate were separated by thin layer chromatography. The radioactivity of individual lipids was measured and expressed as percent of activity incorporated into total lipids.

[b]Values are means ± SEM for four to eight samples. Each sample consisted of livers from one to four animals depending on size.

TABLE III

Partition of [1-^{14}C] palmitate into Complex Lipids, Acetoacetate and CO_2 in the Liver of Developing Rats

Age (day)	Percent incorporation of radioactivity[a]		
	Lipids	AcAc	CO_2
20-day fetus	76.9 ± 4.4[b]	15.4 ± 2.4	7.8 ± 2.1
At birth	79.1 ± 2.5	16.4 ± 2.7	4.5 ± 0.6
1	60.3 ± 4.1	36.1 ± 3.8	3.7 ± 0.4
2	58.0 ± 2.8	37.3 ± 2.5	4.7 ± 0.5
3	55.8 ± 2.8	38.4 ± 2.3	5.8 ± 0.7
5	46.6 ± 3.9	45.7 ± 3.1	7.8 ± 1.2
11	52.0 ± 3.7	41.7 ± 3.7	6.5 ± 0.4
15	50.2 ± 3.3	43.2 ± 3.1	6.6 ± 0.5
27	67.7 ± 2.9	22.2 ± 2.5	9.9 ± 1.1
Adult fed	83.4 ± 2.2	14.9 ± 1.9	1.8 ± 0.3
Adult fasted	40.7 ± 0.8	55.4 ± 1.0	3.9 ± 0.6

[a]Percent incorporation into each fraction was calculated on the basis of total [1-^{14}C] palmitate radioactivity recovered in complex lipids, acetoacetate (AcAc) and CO_2.

[b]Values are means ± SEM for four to eight samples. Each sample consisted of livers from one to four animals depending on size.

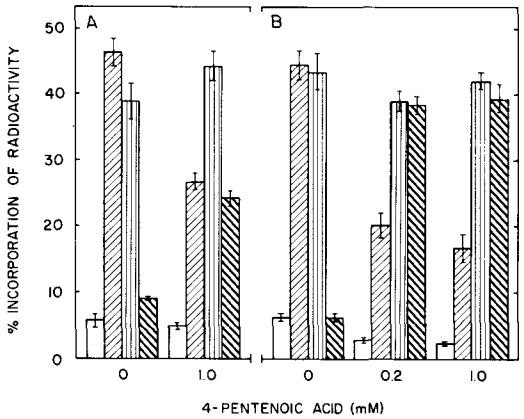

FIG. 4. Effect of 4-pentenoic acid on the partitioning of [1-^{14}C] palmitate into acetoacetate, CO_2 and complex lipids in the liver of developing rats. The liver homogenates prepared from 3-day-old rats (Panel A) and 10-day-old rats (Panel B) were incubated in the same system described in Figure 2. Values are means ± SEM for four samples. The radioactivities of CO_2 (□), AcAc (▨), complex lipids (▥) and nonutilized palmitate (▧) were determined according to the procedures described in "Materials and Methods."

2.4- to 3-fold of adult fed rats. Similarly, the percent of ^{14}C emerging as $^{14}CO_2$ was greater in preparations from suckling rats than from adult fed rats. The percent incorporation of [1-^{14}C] palmitate to lipids, on the other hand, was lower in livers from developing rats than from adult fed rats. At the age of 27 days, 5 days after weaning, the radioactivity incorporated into AcAc decreased with a simultaneous increase in complex lipids. During the suckling period, the proportions of radioactivity from [1-^{14}C] palmitate found in AcAc, CO_2, and lipids were 36-46%, 4-8%, and 47-60%, respectively; whereas, in adult fed rats, 15% of the activity was found in AcAc, 2% in CO_2 and 83% in lipids. In adult fasted rats, the percentage of ^{14}C labeled palmitate incorporated into AcAc was even higher than that of suckling rats, but there was also a proportional decrease in total complex lipids labeled. It should be noted that during the 30-min incubation more than 90% of [1-^{14}C] palmitate was metabolized to lipids, ketones, and CO_2 in homogenates of suckling and adult rats, in contrast to only 55% and 32% incorporation in fetuses and newborns at birth, respectively (data not shown).

To further examine possible regulatory mechanisms, we incubated liver homogenates with 4-pentenoic acid, an inhibitor of fatty acid oxidation and ketogenesis (14) to determine whether lipid synthesis could be enhanced. The partitioning of [1-^{14}C] palmitate into synthetic and degradative processes was measured as previously described, and the results are summarized in Figure 4. In 10-day-old rats, 4-pentenoic acid at 0.2 mM inhibited synthesis of AcAc and CO_2 by about 55%, but it did not enhance lipid synthesis, and consequently increased the level of nonutilized FFA (Fig. 4B). At a higher concentration (1.0 mM), 4-pentenoic acid did not further inhibit AcAc and CO_2 production. In the liver of 3-day-old rats, 4-pentenoic acid had a similar effect on [1-^{14}C] palmitate utilization (Fig. 4A). Although 4-pentenoic acid did not affect CO_2 production, decrease in AcAc and unchanged lipids also resulted in an accumulation of free [1-^{14}C] palmitate. The results of studies obtained from 7-day-old and 15-day-old rats

also demonstrated that the inhibition of ketone production by 4-pentenoic acid did not enhance the synthesis of complex lipids from [1-^{14}C]palmitate (data not shown). By contrast, when 0.2 mM 4-pentenoic acid was incubated with liver homogenates of adult fasted rats, total incorporation of [1-^{14}C]palmitate into: lipid increased from 54.6 ± 3.5 to 70.8 ± 2.2%, AcAc decreased from 40.4 ± 2.2 to 16.5 ± 2.5%, CO_2 decreased from 3.2 ± 0.4 to 0.7 ± 0.1%, and nonutilized labeled fatty acid increased from 1.8 ± 0.2 to 11.9 ± 0.3%.

DISCUSSION

These results demonstrate that the production of [^{14}C]AcAc from [1-^{14}C]palmitate in vitro can be used to estimate total hepatic capacity for synthesis of ketone bodies (Figs. 1 and 2). Under the conditions of these experiments, there was no net accumulation of newly synthesized β-OHB in liver homogenates during incubation unless exogenous fatty acids were added to the incubation medium (Table I). In the presence of an optimal concentration of palmitate, e.g., 0.2 mM, the synthesis of β-OHB accounted for less than 15% of the total ketone bodies synthesized. The net synthesis of β-OHB might be attributed to the increased availability of NADH in mitochondria following β-oxidation of exogenous fatty acid (15). Indeed, McGarry and Foster have reported that both β-OHB and AcAc were synthesized in liver homogenates when NADH was present, but only AcAc was produced in its absence (13).

When expressed as μmole of [1-^{14}C]palmitate incorporated into product/g liver/30 min, the specific activity of [^{14}C]AcAc synthesis represents a relative, but not absolute, capacity for ketone production. This is because only the exogenously added palmitate (0.2 mM) but not endogenous fatty acids in the liver homogenates was included in calculations. However, since the production of complex lipids and CO_2 was expressed in the same manner, [1-^{14}C]palmitate provides a useful means of determining the relative utilization of fatty acids through lipogenic, ketogenic, and oxidative pathways.

We observed that the partition of fatty acids into AcAc increased rapidly after the postnatal feeding and further increased with age. During the mid-suckling period between days 5 and 15, approximately equal portions of available free fatty acids were utilized in the synthesis of complex lipids, and in the production of AcAc and CO_2. By contrast, in the livers of adult fed rats, the amount of fatty acids incorporated into lipids was five times the amount metabolized to CO_2 and KB. In suckling rats, ketone production accounted for most of the increased utilization of fatty acids through the oxidative pathway. However, the increased utilization of fatty acids for ketogenesis is not associated with any impairment in the capacity for synthesizing complex lipids because hepatic synthesis of complex lipids from fatty acids was saturated throughout the suckling period and an inverse relationship was not observed between lipogenic and ketogenic capacities.

Because increased hepatic ketogenesis of fasted rats was accompanied by a proportional decrease in esterification of fatty acids to triglycerides and phospholipids as shown in the present (Fig. 3) and earlier studies (8,16), one might speculate that the cause of newborn ketosis is different from fasting ketosis. The enhanced ketogenesis observed in fasted rats could be, in part, attributed to the competition for fatty acids between esterification and oxidation in favor of the generation of AcCoA (16). This contention is substantiated by the present observation that inhibition of ketone production by 4-pentenoic acid increased synthesis of complex lipids in adult fasted rats. In suckling rats, such a competition for fatty acids is unlikely because the rate of complex lipid synthesis reached a maximum and was not stimulated in the presence of increasing fatty acid concentrations by either addition of exogenous palmitate or inhibition of ketogenesis by 4-pentenoic acid. Further consideration of the increased concentration of fatty acids in the liver (17) and plasma (1) during suckling period leads us to assume that the high-fat content of milk (7) provides sufficient fatty acids for maximal synthesis of both complex lipids and KB in developing rats.

However, increase in fatty acid concentration cannot, by itself, accelerate ketogenesis (1,18). It is generally believed that the rate of ketogenesis is determined by the production of AcCoA and the removal of the CoA derivative (16). Since AcCoA serves as a common precursor for fatty acid synthesis, Krebs' cycle oxidation and ketone production, quantitative changes in one pathway for the utilization of AcCoA can result in alterations of the others. The production of AcCoA, on the other hand, depends on β-oxidation of fatty acids (19) which in turn is under the control of long chain fatty acyl CoA carnitine transferase (20) and the availability of carnitine (21-23). Earlier studies on the development of acyl transferase showed that the activity in suckling rats was two- and threefold of the adult animals (20,24). Recently, Robles-Valdes et al. have reported that livers of suckling rats contained 0.2-0.6 mM carnitine which were two- to six-

fold of the adult values (21). These findings, together with a continuous supply of fatty acids (1), could result in stimulation of β-oxidation and hence, the production of AcCoA. Indeed, it was observed in the present experiment that $^{14}CO_2$ formation from [1-^{14}C]palmitate was enhanced throughout the suckling period. Therefore, it is likely that the high rate of ketogenesis in suckling rats stems in part from the accelerated influx and rapid oxidation of milk fatty acids and a consequent increase in AcCoA production. In addition to its stimulatory effect on fatty acid oxidation (20,22,23), carnitine suppresses the incorporation of fatty acids into complex lipids in liver cells isolated from adult rats fed ad libitum or fasted for 24 hr (22). Thus, it is conceivable that the higher ketogenesis/esterification ratios observed in suckling rats than in adult fed rats is attributable to the elevated levels of endogenous liver carnitine (21).

Consistent with the developmental patterns of ketogenic capacities reported here, Lockwood and Bailey (2) found that activities of ketogenic enzymes, e.g., β-hydroxy-β-methyl glutaryl CoA synthetase and acetoacetyl CoA thiolase were low at birth but increased 15-fold during postnatal development when ketogenesis reached the maximum capacity (Figs. 1 and 2). The involvement of enzymes in ketone production is therefore apparent, although a cause-effect relationship between them has yet to be established. It is important to note, however, that at birth, additions of exogenous palmitate did not stimulate total production of ketone bodies (Table I). This observation, together with the stimulation of ketone production by fatty acids in older rats, suggests that the activity of ketogenic enzymes is a rate-limiting factor for ketogenesis at birth and during early hours of life, whereas the production of AcCoA becomes rate-limiting when enzyme activities have fully developed during the suckling period.

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Erucic Acid and Phospholipids of Newborn Rat Heart Cells in Culture

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ABSTRACT

Erucic acid (Δ^{13}-docosenoic acid), labeled with ^{14}C in the 1- or 14-position, was incorporated into fetal calf serum and fed to beating, neonatal rat myocardial cells in culture. Uptake of the docosenoic acid during the first 6 hr of incubation was 41 nM/hr/mg protein in 7-day old cells and 29 nM/hr/mg protein in 14-day old cells. Fifty-seven percent of the ^{14}C-activity was taken up from the medium in 24 hr, of which 77% was in the cells and 23% was unaccounted for. Of the ^{14}C-activity taken up, 26% was in extractable lipid, with two-thirds in neutral lipid and one-third in phospholipid. Within the neutral lipid fraction, 88% of the ^{14}C-activity was present in triglycerides; while in phospholipids, 66% of the ^{14}C-activity was in phosphatidylcholine (PC); 14% in phosphatidylethanolamine (PE); 6% in sphingomyelin (SPH) and 1% or less in cardiolipin (DPG). PC had the highest specific activity, followed by SPH and PE. The specific activity of PE was one-half that of SPH when the ^{14}C-erucic acid substrate was labeled at the carboxyl position, but increased to equal that of SPH when the substrate was labeled at the double bond. The fatty acids of PC, PE, and SPH were influenced by erucic acid in the growth medium, but the amounts of each phospholipid were not affected. It is proposed that the altered fatty acid composition associated with incorporation of erucic acid or its metabolites into PC, PE, and SPH may affect integrity and function of heart cell membranes.

INTRODUCTION

Erucic acid, a major component in some rapeseed oils, can induce early cardiac lipidosis in rats (1,2) and may be related to myocardial lesions associated with prolonged intake (3-5). Evidence for the incorporation of dietary erucic acid into rat myocardial phospholipids was reported by Blomstrand and Svensson (6), who found that the fatty acids of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin were altered. Erucic acid appeared to have a specific affinity for cardiolipin in rat heart mitochondria.

Studies of erucic acid metabolism in tissue culture preparations of beating, rat heart cells (7,8) showed that this long chain fatty acid is rapidly taken up by the cells and that it is readily incorporated into various lipid classes. Other studies (9) with the isolated, perfused rat heart have also produced evidence for the incorporation of erucic acid or its metabolites into neutral lipid and phospholipid components of the myocardium.

In the present report, we have investigated in further detail the major phospholipid species of beating, neonatal rat myocardial cells maintained in culture with or without erucic acid.

MATERIALS AND METHODS

Cell Culture

Heart cell cultures were prepared by a modification of a method previously described (10). From 36 to 40 newborn, male and female Wistar rats, age 3 to 5 days (Bio-Breeding Labs, Ottawa, Canada), were used. Ventricular tissue from excised hearts was digested in a magnetic stirrer (Wheaton Celstir, 200 rpm) with 15 ml of a sterile, 0.1% solution of trypsin (Calbiochem, Elk Grove Village, IL, B grade, 1x crystalline, activity: 2500 NF units per mg protein) dissolved in Tyrode's solution (free of Ca^{++} and Mg^{++}). The trypsin was first discarded following each of two 15 min cycles of digestion and then retained after each of eight 25 min cycles. The cells dispersed in trypsin solution were recovered by centrifugation (600 x g, 5 min); resuspended in Eagle's MEM with Earle's balanced salt solution and 10% fetal calf serum, GIBCO No. 614, penicillin 100 mcg/ml, and streptomycin, 100 units/ml; then plated in plastic culture dishes (Falcon No. 3002, 60 mm) at a seeding density of ca. 3×10^6 cells per dish. Incubation was at 37 C (95% air and 5% CO_2). The medium was changed after 24 hr and every 2 days thereafter. The cultures became confluent within 48 hr and were used for experiments after 7 or 14 days of growth. Protein in cell cultures was measured by the Hartree (11) modification of the method of Lowry et al. (12).

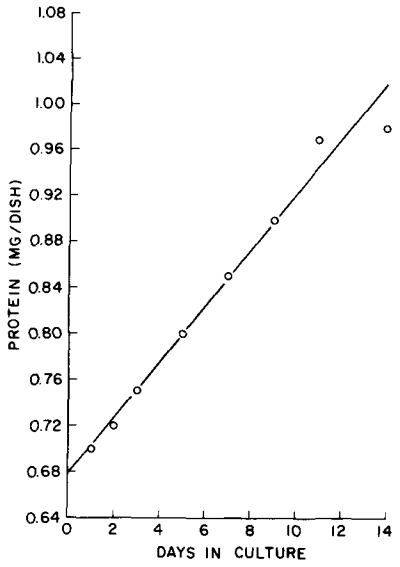

FIG. 1. Relationship between protein concentration of heart cells and number of days in culture.

Lipid Analyses

Lipid was extracted from heart cell cultures by the method of Bligh and Dyer (13), and prior to use, was stored at -20 C in chloroform containing 0.1% methanol. Phospholipids were separated from total lipid extracts by thin layer chromatography (TLC) on Silica Gel H (E. Merck, Darmstadt, Germany) employing the conditions described previously (16). Phospholipid fractions for later analysis were recovered from silica gel by aspiration with 5 ml of a solution of 1 N HCl in methanol (1:19 v/v) according to Dodge and Phillips (17). Phosphorus in chloroform extracts of lipid and in phospholipids from thin layer chromatograms was determined by the method of Bartlett (14) as modified by Parker and Peterson (15). Fatty acids of the phospholipids separated by TLC were analyzed in a Perkin-Elmer gas chromatograph, model 990, equipped with a 50 ft stainless steel S.C.O.T. column (ID = 0.2 mm) internally coated with diethylene glycol succinate. The carrier gas was helium (flow rate, 2 ml/min) and the oven temperature 180 C (isothermal). Phosphatidylcholine and phosphatidylethanolamine were subjected to alkaline hydrolysis by refluxing 5 min in 0.5 N NaOH in methanol followed by transmethylation of the liberated fatty acids in 14% boron trifluoride in methanol (18). The fatty acids of sphingomyelin were prepared by the method of Kates (19).

Neutral lipids were separated from total lipid extracts by TLC on Silica Gel G (0.4 mm, E. Merck, Darmstadt) coated on glass strips (20 x 5 cm). The method involved the sequential use of 2 solvent systems in a modification of the procedure of Hojnacki and Smith (20). Each plate was developed to a height of 12 cm above the origin in solvent 1 (pentane-diethyl ether-glacial acetic acid, 80:20:1 v/v), then air-dried 10 min at room temperature and redeveloped in the same direction in solvent 2 (pentane-diethyl ether, 97:3 v/v) to a height of 1 cm from the top of the plate. All samples were run along with a standard mixture of six neutral lipid classes (20) co-chromatographed on a second glass strip. Lipid spots were visualized with I_2 vapors or by sulphuric acid-dichromate spray followed by charring at 180 C for 10 min. Neutral lipid and phospholipid components for measurement of radioactivity, were recovered quantitatively from silica gel plates by the method of Kritchevsky and Malhotra (21).

Radioactive Substrates

Erucic acid was purchased from CEA, Saclay, France as the carboxyl ^{14}C-labeled compound (specific activity, 40 μCi/μM) or as the [14-^{14}C]-labeled compound (specific activity, 47 μCi/μM). The labeled fatty acids were diluted with unlabeled erucic acid (Sigma Chemical Company, St. Louis, MO) so that the ^{14}C-labeled fatty acid constituted 1.8% of the total erucic acid (8). After conversion to the K^+ soap by incubation 5 min in alcoholic KOH at 50 C, the total erucate was dispensed aseptically into 100 ml fetal calf serum (GIBCO No. 614). The serum plus erucate was diluted to 20% v/v in Eagle's MEM medium to yield a final concentration of total erucate of 0.2 μM per ml of medium or 1 μM per culture dish (5 ml).

Radioactivity in culture media, in lipid extracts in $CHCl_3$, or in phospholipids and neutral lipids separated by thin layer chromatography (TLC), was measured in a Packard liquid scintillation spectrometer (Model 3003). An aliquot of sample was counted in 10 ml of Aquasol (New England Nuclear Corporation, Boston, MA) except when counting in the presence of silica gel (21), in which case 15 ml of Aquasol were used. All counts were corrected by external standardization, and specific activity given as counts per minute per microgram of lipid phosphorus.

RESULTS

Growth in tissue culture was estimated from the change in total cell protein during incubation. The relationship between protein concentration and the number of days in culture is shown in Figure 1. A linear increase in tissue

protein occurred during the 14 day incubation period, and was unaffected by inclusion of erucic acid (1 μM/culture dish) in the medium.

The rate of uptake of [14-^{14}C]-labeled erucic acid by 7- and 14-day old cultures of beating rat heart cells is compared in Figure 2. At zero time, the concentration of erucic acid per culture dish was 1 μM. Uptake of this fatty acid, in nM/mg protein, was essentially linear during the first 6 hr of incubation but showed a slower rate of increase in the next 18 hr. The estimated rate of uptake during the first 6 hr was 41 nM/hr/mg protein in 7-day old cells but only 29 nM/hr/mg protein in 14-day old cells.

The distribution of ^{14}C-activity in 7-day old heart cell cultures after 24 hr incubation was [14-^{14}C]-labeled erucic acid is shown in Table I. Some 57% of the ^{14}C-activity was taken up from the medium in 24 hr, of which 77% was in the cells and 23% was unaccounted for. About 51% of the ^{14}C-activity taken up by the cells was in nonlipid fraction and 26% was in extractable lipid, of which 64% was in neutral lipid and 36% in phospholipid.

Thin layer chromatography was used to separate the neutral lipids of heart cells incubated 24 hr with [14-^{14}C]-labeled erucic acid. The distribution of ^{14}C-activity among the major neutral lipid classes is shown in Table II. Most of the lipid radioactivity was located in the triglyceride fraction. In this solvent system, phospholipids remained at the origin. Total recovery of radioactivity was 98% of which 36% was in phospholipids and 64% in neutral lipids.

Up to one-third of the lipid radioactivity was consistently present in the phospholipid fraction. Therefore, it was of interest to establish the distribution of erucic acid or its metabolites among individual heart cell phospholipids.

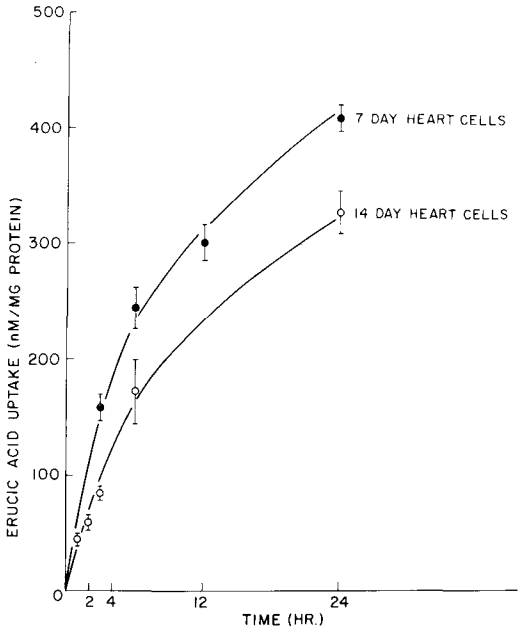

FIG. 2. Relationship between erucic acid uptake and incubation time in hours for 7- and 14-day old cultures of beating, rat heart cells.

Seven day old heart cell cultures were incubated 24 hr with [14-^{14}C]-labeled erucic acid, then extracted of lipid and the ^{14}C-activity in the various phospholipid fractions determined after separation by TLC. As shown in Table III, phosphatidylcholine was the most abundant phospholipid (40% of total P) and contained 66% of the ^{14}C–activity. The second most abundant phospholipid was phosphatidylethanolamine (25% of total P) but contained only 14% of the radioactivity. Other ^{14}C-activity values were sphingomyelin 6%, lyso-

TABLE I

Distribution of ^{14}C-Activity in Heart Cells Incubated 24 hr with [14-^{14}C]-Erucic Acid[a]

Location	^{14}C-activity DPM x 10^6	%
1. MEM[b], before use	24.0	100
2. MEM after incubation 24 hr	10.4	43 of 1
3. Uptake from MEM in 24 hr	13.6	57 of 1
4. Cells after 24 hr	10.5	77 of 3
5. Uptake lost in 24 hr	3.1	23 of 3
6. Nonlipid fraction, 24 hr	6.9	51 of 3
7. Extractable lipid, 24 hr	3.6	26 of 3
8. Neutral lipid, 24 hr	2.3	64 of 7
9. Phospholipid, 24 hr	1.3	36 of 7

[a]Total erucic acid concentration = 1 μM/culture dish. Age of cells in culture = 7 days.
[b]MEM = minimum essential medium.

TABLE II

^{14}C-Activity in Major Neutral Lipid Classes of Heart Cells Incubated 24 hr with [14-^{14}C]-Labeled Erucic Acid

Neutral lipid	DPM/Fraction[a]	^{14}C-activity in neutral lipid %
Solvent front	1,918	1.2
Cholesteryl esters	9,264	5.7
Triglycerides	142,890	87.9
Free fatty acids	3,609	2.2
Cholesterol	4,801	3.0
Total neutral lipid DPM =	162,482	

[a]Total DPM applied to TLC plate = 258,842. Total DPM recovered from TLC was 253,396 (or 98%) of which 64% was neutral lipid and 36% was phospholipid (DPM at origin = 90941).

TABLE III

Phospholipid Phosphorus Values and Phospholipid Radioactivity in Heart Cells Incubated with Unlabeled- and ^{14}C-Labeled Erucic Acid

Phospholipid spot[a]	Phospholipid phosphorus % of phosphorus recovered		Phospholipid ^{14}C-activity[d]	
	Control	22:1[c]	DPM/spot	% of total DPM
	(3)	(3)		
DPG	5.7 ± 0.1	6.6 ± 0.7	2,000	0.9
PA	5.2 ± 1.2	3.4 ± 0.5	4,994	2.4
PE	25.1 ± 0.5	25.5 ± 1.1	29,194	13.8
PS + PI	12.9 ± 0.1	12.0 ± 0.3	21,432	10.1
PC	39.2 ± 1.1	40.5 ± 0.3	138,800	65.7
SPH	6.9 ± 0.4	6.9 ± 0.4	12,266	5.8
LPC	2.7 ± 0.2	2.4 ± 0.2	2,334	1.1
Origin	1.8 ± 0.1	0.9 ± 0.1	384	0.2
		Total DPM =	211,404	

[a]Abbreviations: DPG, diphosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

[b]Mean ± standard error of the mean of (3) determinations.

[c]Unlabeled erucic acid = 1 μM/dish. Incubation time was 96 hr.

[d]Age of cells in culture was 7 days. Incubation time with [14-^{14}C]-erucic acid was 24 hr.

phosphatidylcholine 1.1%, and cardiolipin at 1% or less. Erucic acid was without effect on the amounts of individual heart cell phospholipids.

To determine if differences exist among the major phospholipids in the uptake of erucic acid or its metabolites, the specific activities of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin were measured following incubation of heart cell cultures for 24 hr with [1-^{14}C]- or [14-^{14}C]-labeled erucic acid (Table IV). Figures in parentheses are the specific activity values for each phospholipid normalized to that of phosphatidylcholine which was arbitrarily set at 100. The specific activity of phosphatidylethanolamine (Exp. 1 and Exp. 2) was one-half that of sphingomyelin when the substrate was labeled at the carboxyl group, but was equal to that of sphingomyelin when the substrate was labeled at the double bond.

The fatty acid composition of phosphatidylcholine, phosphatidylethanolamine and sphingomyelin was determined before and after incubation with erucic acid (Table V). After 96 hr, erucic acid was 9% of the fatty acids of phosphatidylcholine and 5% of those of phosphatidylethanolamine, but less than 2% of the fatty acids in sphingomyelin. In the presence of erucic acid, the proportions of palmitic, stearic, and arachidonic acids decreased in phosphatidylcholine and in phosphatidylethanolamine while that of oleic acid increased. With the exception of an increase in the proportion of behenic acid (22:0), erucic acid was without effect on the relative proportions of fatty acids

TABLE IV

Specific Activity of Phospholipids from Heart Cells Incubated 24 hr with [1-^{14}C]- or [14-^{14}C]-Erucic Acid

Phospholipid[b]	Specific activity x 10^{-2} [a]	
	Erucic acid 1-^{14}C	Erucic acid 14-^{14}C
	Exp. 1	
SPH	42 (53)[c]	66 (31)
PC	79 (100)	210 (100)
PE	19 (24)	66 (31)
Ratio: SPH/PE	2.2	1.0
	Exp. 2	
SPH	66 (60)	50 (30)
PC	111 (100)	169 (100)
PE	36 (32)	52 (31)
Ratio: SPH/PE	1.8	1.0

[a]Specific activity = DPM/min/μg P.

[b]For abbreviations: see Table III.

[c]Figures in parentheses denote specific activity relative to that of phosphatidylcholine arbitrarily set at 100. Age of cells in culture was 7 days.

TABLE V

Major Fatty Acids of Phosphatidylcholine, Phosphatidylethanolamine, and Sphingomyelin in Rat Myocardial Cells before and after Incubation with Erucic Acid

Fatty acid	Major fatty acids, weight percent values[a]					
	Phosphatidylcholine		Phosphatidylethanolamine		Sphingomyelin	
	Control	Erucic[b]	Control	Erucic	Control	Erucic
16:0[c]	(3)	(3)	(3)	(3)	(3)	(3)
16:0[c]	33.7 ± 1.3[d]	24.7 ± 1.2**[e]	7.9 ± 1.0	6.7 ± 0.7	37.7 ± 4.2	38.6 ± 1.9
18:0	14.8 ± 0.5	8.8 ± 0.3**	25.4 ± 1.1	19.2 ± 1.6*	16.6 ± 0.9	12.1 ± 1.6
18:1	23.3 ± 1.9	31.6 ± 1.7*	11.0 ± 0.4	19.9 ± 0.5**	7.0 ± 5.2	10.4 ± 5.8
18:2	3.1 ± 0.2	2.6 ± 0.1	1.4 ± 0.3	2.1 ± 0.5	1.1 ± 0.1	1.1 ± 0.1
20:1	-	3.6 ± 0.5	-	2.9 ± 0.7	-	tr
20:2	2.5 ± 0.6	4.0 ± 1.3	1.4 ± 0.4	3.1 ± 1.2	-	-
20:4	11.3 ± 0.6	8.1 ± 0.5**	28.5 ± 3.7	23.9 ± 1.8	-	-
22:0	-	-	tr	-	5.0 ± 0.8	9.2 ± 0.8**
22:1	-	8.7 ± 0.5	-	5.2 ± 0.1	-	1.8 ± 0.6
22:5	tr	tr	3.6 ± 0.5	2.2 ± 0.1*	tr	-
22:6	1.2 ± 0.2	1.1 ± 0.1	5.8 ± 0.7	2.9 ± 0.5*	tr	tr
24:0	-	-	-	-	13.7 ± 3.1	12.6 ± 1.7

[a]Also contained minor to trace (tr) amounts of 14:0, 15:0, 16:1, 17:0, 18:3, 20:0, and 20:3.

[b]Erucic acid conentration = 1 μM/culture dish. Incubation time was 96 hr.

[c]Number of carbon atoms: number of double bonds.

[d]Mean ± standard error of the mean of (3) determinations.

[e]**Significantly different from the control value at $p<0.01$. *Significantly different from the control value at $p<0.05$.

in sphingomyelin.

DISCUSSION

A 30% decline in the rate of uptake of erucic acid by 14-day old cells compared to 7-day old cells reflected the gradual dedifferentiation of neonatal rat heart cells in culture (23). The amount of erucic acid taken up by the older cells was, however, threefold greater than previously reported under different experimental conditions (8).

The linear increase in tissue protein that occurred during a 2 wk incubation period was supported by the earlier observations of Orloff and McCarl (24) and Pinson (8), but differed from the reported growth characteristics of heart cells following myoblastic enrichment (7,22). The present findings are in conflict with earlier data (8) which suggested that erucic acid

may enhance protein synthesis by rat heart cells in culture. Unpublished work from our laboratory has shown that erucic acid did not alter the ability of the heart cells to utilize amino acids.

Pinson and Padieu (22) showed that rat heart cells in culture can rapidly metabolize ^{14}C-erucic acid with the release of $^{14}CO_2$. In other work (7), less than 10% of the total lipid ^{14}C-activity was detected in phospholipids of 7-day old cells. This was in contrast to the present findings in which the phospholipid fraction contained up to 36% of the lipid ^{14}C-activity.

The presence of a large amount of lipid radioactivity in the triglyceride fraction from heart cell cultures following incubation with ^{14}C-labeled erucic acid confirmed the findings of Pinson and Padieu (7) and suggested a parallelism with the condition of early lipidosis seen in the myocardium of rats fed diets rich in erucic acid (1,2).

The phosphatidylcholine fraction contained the largest percentage of the total phospholipid ^{14}C-activity after incubation of 24 hr. This was at variance with earlier work (7) in which sphingomyelin was reported to be the most heavily labeled phospholipid following incubation of heart cell cultures with ^{14}C-erucic acid. These earlier values may have arisen, in part, from incomplete phospholipid separation during TLC.

Carroll (26) concluded that transformation of erucic acid to longer or shorter chain metabolites preceded its incorporation into liver phospholipids. Other studies have shown that the stepwise degradation of erucic acid to eicosenoic acid and oleic acid can take place in the intact rat (27) and in rat myocardial cell culture (8,22). As shown here, uptake of erucic acid into phosphatidylcholine, phosphatidylethanolamine and sphingomyelin of heart cell cultures was less than would be expected from radioactivity values.

In studies (4,28) with rats fed erucic acid, only small amounts were incorporated into cardiac phospholipids. Blomstrand and Svensson (6), working with pooled heart mitochondria from rats fed rapeseed oil, detected erucic acid in phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. In the present study, neonatal rat heart cells showed similar behavior, resulting in the incorporation of this fatty acid into phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin (Table V). Cardiolipin contained less than 1% of ^{14}C-activity derived from labeled erucic acid substrates and was not examined by gas liquid chromatography (GLC).

The low level of erucic acid in sphingomyelin and the high level of ^{14}C-activity associated with this phospholipid suggested that sphingomyelin may preferentially incorporate shorter chain metabolites of this long chain fatty acid. If one assumes that β-oxidative degradation, with the accompanying loss of CO_2, precedes the incorporation of erucic acid, or its metabolites, into sphingomyelin, then this could explain, in part, the low specific activity of phosphatidylethanolamine, relative to that of sphingomyelin, when the substrate was labeled at the carboxyl group, while the specific activities were equal when the substrate was labeled at the double bond. The overall lower specific activities obtained with carboxyl-labeled erucic acid (Table IV) can be attributed to the rapid loss of $^{14}CO_2$ from heart cell cultures when incubated with this ^{14}C-labeled docosenoic acid (22).

The presence of erucic acid or its metabolites in heart phospholipids was accompanied by changes in the fatty acid pattern in phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin. However, the amounts of phospholipids, while unaffected by erucic acid, were in agreement with our previous findings (10) and with the values reported for rat heart muscle by Simon and Rouser (29). The occurrence of erucic acid in phosphatidylcholine and in phosphatidylethanolamine was accompanied by an increase in oleic acid. A similar oleic acid effect has been shown to occur in phospholipids of rats fed diets rich in erucic acid (30). In the absence of erucic acid, the fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin resembled values reported for the subcellular fractions of human (31) myocardial tissue.

Under our experimental conditions, erucic acid was taken up by cells of the rat myocardium in tissue culture and, along with its metabolites, readily incorporated into specific neutral lipid and phospholipid components of the cardiac cells. The structural role of phospholipids in mammalian cell membranes and its relationship to phospholipid fatty acid composition has been recognized (32). The altered fatty acid composition associated with the incorporation of erucic acid or its metabolites into phosphatidylcholine, phosphatidylethanolamine, and sphingomyelin, therefore, may have important effects on integrity and function of heart cell membranes.

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Delayed Conversion of Squalene to Sterols during Development of *Pinus pinea* Seeds

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ABSTRACT

During germination of seeds of the gymnosperm, *Pinus pinea*, radioactivity from [2-^{14}C]-mevalonate proceeded principally through the anaerobic reactions leading to squalene in the first 24 hr in both the haploid endosperm and the diploid embryo, and only with succeeding time (3-9 days) in both cases was label transferred to sterols in oxygen-requiring steps. The rates of turnover must be real and independent in the two tissues, since no consequential interchange of labelled lipids occurred between the endosperm and the embryo. Similar delayed conversion of squalene to sterols has been observed previously during germination of seeds of the angiosperm, *Pisum sativum*.

INTRODUCTION

In earlier work (1,2) on the kinetics of the biosynthesis of sterols, it was shown that the enzymes for the anaerobic steps from mevalonate to squalene were very active during the entire period of germination of seeds of the angiosperm, *Pisum sativum*, but that activity in the enzymes for succeeding steps, which include aerobic ones, (epoxidation of squalene, removal of methyl groups, etc.) developed only with time. During the first 24 hr of germination, label from [2-^{14}C]-mevalonate proceeded primarily to squalene. However, when preincubated with water for 24 hr and then presented with [2-^{14}C]-mevalonate, label proceeded primarily to the pentacyclic triterpenoid β-amyrin, but only after three or more days of preincubation (with water) did label from [2-^{14}C]-mevalonate appear appreciably in sterols (1). In order to ascertain whether this developmental phenomenon reflected a generality, which speculatively might be associated with evolutionary recapitulation (1-3), we examined the influence of development on the biosynthetic kinetics of germinating seeds of a gymnosperm (*Pinus pinea*). The sterols of both the diploid embryo and the encapsulating haploid gametophyte (endosperm) of *P. pinea* as well as of the whole seed of *P. sativum* have been examined and shown to be the same (24α-ethyl-, 24α-methyl- and 24β-methylcholesterol) (4-6). The results of the present study show that in the gymnosperm a delay in the appearance of label in these sterols occurs which is entirely analogous to the previous observations in the angiosperm. Moreover, the delay occurred independently in both the embryo and the endosperm with no consequential transfer of lipids from one to the other.

EXPERIMENTAL PROCEDURE

Fresh, intact seeds of *Pinus pinea*, the Italian stone pine, were obtained from Herbst Brothers Seedsman, Inc., 1000 N. Main St., Brewster, NY, 10509. Racemic [2-^{14}C]-mevalonic acid as the dibenzylethylenediamine salt (New England Nuclear Corp., Boston, MA) was used for all incubations with the pine seeds. Cycloartenol was isolated from *Strychnos nux-vomica*. Cholesterol of high purity was purchased from Nutritional Biochemicals (Cleveland, OH) and used without further purification.

P. pinea seeds were sterilized and prepared for germination as described previously (5,7). One group of seeds was presented with [2-^{14}C]-mevalonate in the water of imbibition. The [2-^{14}C]-mevalonate solution containing 1.10 μCi (50 μg) in 1 ml sterile, distilled water was pipetted over 30 seeds. Within 5 hr the solution was completely absorbed. The swollen seeds were transferred to sterile Petri dishes lined with moistened filter paper for the remainder of the germination period. Groups of 30 seeds were germinated for 1, 3, 5, 7, or 9 days after imbibition. Sterile, distilled water was added as necessary to maintain a moist environment. By the fifth day of growth, the leaves were a medium green color with a slight "piney" odor. Roots varied from 1 to 5 mm in length. The leaves turned dark green by day 9 and the roots attained a length of 3 to 4 cm. A second group of seeds was germinated in the presence of distilled water for 5 days. Batches of 13 or 14 of the 5-day germinated seeds were placed in sterile Petri dishes on filter paper moistened with a 1 ml solution containing 1.10 μCi (50 μg) of [2-^{14}C]-mevalonate or on gelled agar containing the same amount of [2-^{14}C]-mevalonate. These

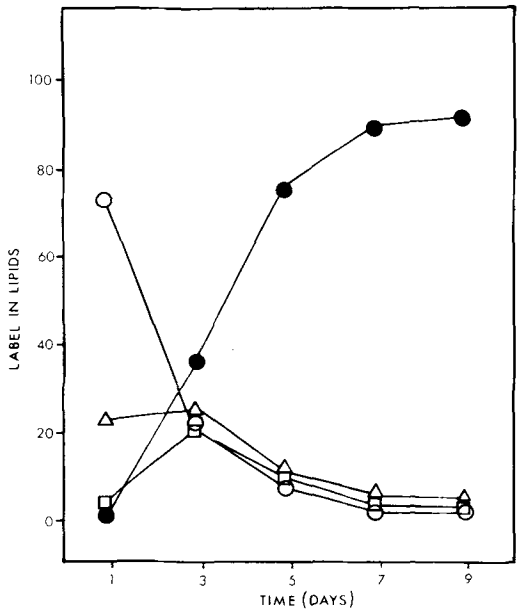

FIG. 1. The effect of time on the appearance of ^{14}C from [2-^{14}C]-MVA in squalene (○—○), the 4,4-dimethylsterols (△—△), the 4α-methylsterols (□—□), and the 4-desmethylsterols (●—●) of *Pinus pinea* gametophyte.

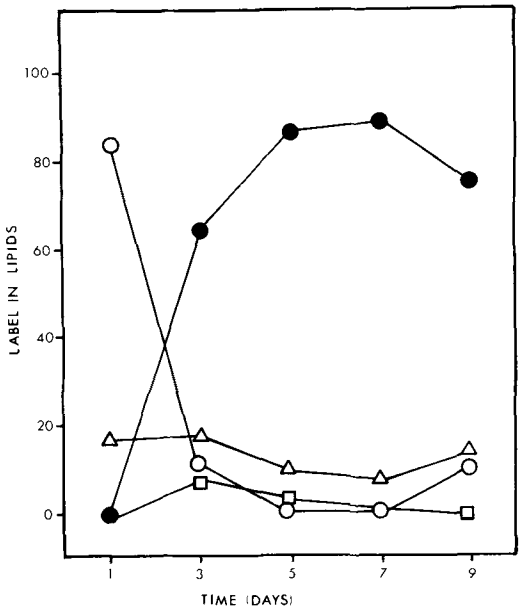

FIG. 2. The effect of time on the appearance of ^{14}C from [2-^{14}C]-MVA in squalene (○—○), the 4,4-dimethylsterols (△—△), the 4α-methylsterols (□—□), and the 4-desmethylsterols (●—●) of *Pinus pinea* embryo.

seeds were germinated for another one or two days to allow the substrate to enter the seeds primarily through the roots. The group growing on agar rapidly extended their roots directly into the agar, and root hairs were clearly visible. The group growing on filter paper had their roots firmly in contact with the paper as a result of normal geotropic growth habit.

At the end of the incubation periods, the embryo (seedling) was surgically detached from the white female gametophyte (endosperm). In many cases, a gummy fluid was observed in the embryo cavity. The tissues were placed in separate mortars and ground with a small amount of sand and 10% ethanolic KOH (w/v). The resulting pastes were transferred to separate round bottom flasks and refluxed with 100 ml of ethanolic KOH for 30 min. The cooled mixtures were diluted and extracted with diethyl ether to yield the neutral lipids.

The embryo and female gametophyte neutral lipids were subjected to chromatography on a thin layer (0.25 mm) of silica gel developed twice in $CHCl_3$. The plates were scanned for radioactivity on an Atomic Accessories, Model RCS-363, radiochromatogram scanner, and regions with the same R_F as squalene, cycloartenol, and cholesterol were eluted into liquid scintillation vials and counted in a Nuclear Chicago, Series 720, Liquid Scintillation System. The region between the 4,4-dimethylsterol and the 4-desmethylsterol bands on thin layer chromatography (TLC) was taken to be the 4-methylsterol region.

RESULTS

As shown in detail in Figures 1 and 2, label from [2-^{14}C]-mevalonate appeared primarily in squalene during the first day of germination (24 hr after seeds were presented with water containing the radioactive substrate) in both embryo (83.5% of the embryo neutral lipid) and endosperm (73.0% of the endosperm neutral lipid) which were examined separately. Little or no 4-desmethylsterol was formed (1.9% or 0% of the endosperm or embryo neutral lipid, respectively), but some of the squalene was converted to 4-methyl- and 4,4-dimethylsterols. During the next 4 to 5 days, there was an increasing proportion of label which appeared in the 4-desmethylsterols (90.0% or 89.2% of the endosperm or embryo neutral lipid, respectively, on day 7), a sharp decline in the labelled squalene to 1.2% or 1.4% of the endosperm or embryo neutral lipid, and a slight drop in the amount of radioactive 4-methyl- and 4,4-dimethylsterols.

Since it was possible that the apparent similarity in the behavior of the two tissues

TABLE I

Embryo and Female Gametophyte Neutral Lipids formed from [2-^{14}C]-Mevalonate Presented at Imbibition during 1 to 9 Days of Germination

Day of incubation	Gametophyte Neutral lipids dpm	% of total Neutral lipids[a]	Embryo Neutral lipids dpm	% of total Neutral lipids[a]
1	426,000	91.2	41,400	8.8
3	438,000	91.2	42,400	8.8
5	337,200	93.4	23,600	6.6
7	490,000	91.6	45,400	8.4
9	362,800	92.6	31,000	7.4

[a]Total neutral lipids is the sum of radioactivity in the neutral lipids of the embryo and of the female gametophyte.

TABLE II

Incorporation of [2-^{14}C]-Mevalonate into Embryo and Female Gametophyte Neutral Lipids when the Substrate is Presented after Radicle Emergence (5-Day-Old Seeds)

Incubation conditions	Gametophyte Neutral lipids dpm	% of total Neutral lipids[a]	Embryo Neutral lipids dpm	% of total Neutral lipids[a]
Agar				
24 hr	26,000	14.9	149,000	85.1
48 hr	31,000	15.1	173,000	84.9
Filter paper				
24 hr	40,000	27.6	105,000	72.4
48 hr	53,000	20.9	201,000	79.1

[a]Total neutral lipids is the sum of radioactivity in the neutral lipids of the embryo and of the female gametophyte.

could be an artifact of equilibration of the lipids between them, we examined the problem of transport in the following way. Radioactivity from a single dose of [2-^{14}C]-mevalonate which entered the total neutral lipid pool was measured as a function of time in the two tissues, time being taken from the onset of germination when the substrate was administered. The absorption of substrate in this case has to be by transfer through the outer wall of the endosperm, through the endosperm, and finally to the inner embryo. The endosperm has the larger mass. One would, therefore, expect the endosperm to contain most of the label in the early stages. If some sort of active transport then directed lipids formed from the mevalonate into the embryo, an increasing proportion of label should appear in the embryo in the later stages of germination. In fact, as shown in Table I, more than 90% of the label was in the endosperm neutral lipids at all times contraindicating any transfer of lipids between the tissues. The reverse experiment was also done, i.e., the embryo was presented first with the lable. This was accomplished by germination of the seeds in water until functional roots had appeared on the embryos. The labelled substrate ([2-^{14}C]MVA) was then allowed to enter (primarily) through these roots. This should have and, in fact, did (Table II) produce a distribution of label which was the converse of the one in which substrate first entered the endosperm. In the root-fed seedlings, ca. 80% of the label appeared in embryo lipids during the first 24 hr and there was no significant change during the next 24 hr. Consequently, we believe little or no transport of lipids can be occurring between the two tissues, and the observed delays in the metabolism of squalene in the two tissues must reflect parallel and independent kinetic phenomena in each of them.

DISCUSSION

There are two explanations for the delay in the metabolism of squalene. One is that newly biosynthesized and, therefore, labelled squalene enters a pool of this intermediate which is maintained at a given substantial level. In such a case, label would enter squalene rapidly, become trapped, and be removed only slowly. This explanation assumes that all of the enzymes for the entire pathway to 4-desmethylsterols are present and active and that the

observed delay is due to the trapping effect in the squalene pool. The second explanation assumes that the squalene pool is not large, that it is turned over rapidly, and that the observed rates are derived from the concentrations of active enzymes which are changing with development of the organism. Insufficient information is available to discriminate between these alternatives, but in the earlier work with *Pisum sativum* (1), the manner in which the experiments were carried out (preincubation with water) requires, should the first explanation hold, that the squalene pool must have rapidly decreased during germination. While this could have happened, it is not clear why.

We tentatively think a more attractive albeit speculative answer to the questions posed by delayed metabolism of squalene has to do with the second explanation discussed above and more specifically with the reasons why enzymatic activities might change with development. While the biosynthesis of squalene is entirely nonoxidative, the first and many subsequent steps in its conversion to steroidal endproducts require molecular oxygen. Since there is thought to have been a timed sequence from an earth lacking atmospheric oxygen to one with oxygen, an ontogenetic recapitulation in seeds of a phylogeny dependent on environmental changes should include the early development of anaerobic processes, e.g., the biosynthesis of squalene, and a later development of mixed function oxidases, e.g., those for the conversion of squalene to its epoxide and of cycloartenol to 4-desmethylsterols.

It is interesting to note incidentally that the gametophytic tissue (endosperm) of seeds of the genus *Pinus* has been supposed to function as a nutrient reserve for the embryo. Although movement of carbohydrate from the endosperm into the embryo cavity of the seed has been documented (8), other evidence for a reserve role is circumstantial (9-13). The failure demonstrated in the present paper of isopentenoidal lipid to pass between the endosperm and embryo lends no additional support to the reserve hypothesis.

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Influence of Fasting-refeeding and Dietary Linoleate on Liver Glucose-6-Phosphate Dehydrogenase and Phospholipid Fatty Acid Composition in Rats Adapted to a Purified Diet

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ABSTRACT

Responses to refeeding after fasting were studied in male rats fed a purified casein-sucrose diet containing 5% safflower oil. After a 48 hr fast, the rats were fed either the same diet or the same diet minus oil (fat-free diet). These experiments were designed to distinguish changes due to fasting and refeeding alone without a change of diet from those changes caused by refeeding a diet of different composition. In the first experiment, rats were refed for 3 or 7 days. In rats refed either diet, liver glucose-6-phosphate dehydrogenase (G6PD) activity was elevated above refasting levels, but after 7 days, activity in rats refed the 5% safflower oil diet was significantly lower than in those refed the fat-free diet. The amount of liver arachidonate in rats refed the safflower oil diet was the same during refeeding as before fasting. In the second experiment, rats were refed the fat-free diet for 1, 2, 3, or 7 days. Liver G6PD and fatty acid synthetase were measured, as well as fatty acids in liver total lipids and phospholipids. G6PD activity increased above prefasting levels after one day refeeding and continued to increase for 7 days. Fatty acid synthetase activity increased for the first 3 days of refeeding, with no additional increase after 7 days. In all rats refed the fat-free diet, the proportions of arachidonate and linoleate in liver phospholipids diminished with time, and eicosatrienoate appeared. These results show that (a) maintenance of liver phospholipid arachidonate did not prevent increased G6PD activity in early refeeding, but the elevated G6PD activity later declined when phospholipid arachidonate was maintained by feeding a source of linoleate; (b) the metabolic state of fasted-refed rats had not returned to prefasting conditions even after 7 days of refeeding a linoleate-rich diet to which the rats were adapted before fasting.

INTRODUCTION

Refeeding of a high carbohydrate diet to fasted rats has often been used as a technique for studying lipogenesis in liver (1-5). This procedure stimulates de novo synthesis of fatty acids and increases the activities of lipogenic enzymes, i.e. glucose-6-phosphate dehydrogenase, fatty acid synthetase, acetyl-CoA carboxylase, malic enzyme, and citrate lyase (2,6). The percentages of arachidonate and linoleate in liver total fatty acids decline sharply, even when the prefasting diet contains adequate linoleate (1,2). These phenomena suggested that essential fatty acid (EFA) deficiency could be produced rapidly by fasting-refeeding (1) and that essential fatty acids may have a unique role in regulation of lipogenesis (1,2,6-9).

In fasting-refeeding experiments, two processes can be observed. One is the response of the animal to refeeding after a fast, and the other is the influence of diet composition on the animal's response to fasting-refeeding (10,11). Since responses to fasting-refeeding can be very extreme (fivefold elevations in enzyme activities) it is important to separate, experimentally, these effects from those caused by changes in dietary composition, which are often much smaller. Thus, if the responses to fasting-refeeding are to be studied, the appropriate prefasting diet should be identical to the refed one. If the influence of individual dietary components is sought, then only one component at a time should be changed in the diet refed after fasting (12).

In many of the refeeding studies dealing with lipogenesis, rats have been fed a non-purified chow diet before fasting (1,2,13). The changes during refeeding, therefore, represent adaptation to a high-carbohydrate purified diet as well as to refeeding after fasting. Often these chow-fed rats are referred to as "controls," but clearly they are not proper controls for studying the influence of dietary composition on fasting-refeeding responses. For example, it has been shown that activities of lipogenic enzymes are significantly lower in chow-fed rats than in rats fed a complete purified diet (10). This clearly illustrates that chow-fed rats cannot be accurately compared with rats fed a

purified diet in experiments of this type.

In investigations on fasting-refeeding, it is important to adapt the experimental animals to an adequate purified diet before fasting and then to refeed the identical diet. In this way, one can measure the response to fasting-refeeding without complication by changes in dietary composition. When this response is known, then it is possible to measure changes in response caused by altering individual dietary components, in this case essential fatty acids. We know of no data on lipogenesis in fasted-refed rats that have been adapted in advance to a nutritionally adequate purified diet and then refed the same diet with the fat component removed. Information on such animals is needed to evaluate more definitely the role of EFA in regulation of lipogenesis.

To obtain information on liver lipogenesis under controlled dietary conditions, we have adapted rats to an adequate, purified diet containing 5% safflower oil. The rats were then fasted for 48 hr and refed either with the identical diet or with the same diet without the oil. Fatty acids of liver total lipids and liver phospholipids were measured before and after fasting and during 1-7 days of refeeding in all rats. Liver glucose-6-phosphate dehydrogenase (G6PD) was measured in all rats, and liver fatty acid synthetase (FAS) was measured in rats refed the fat-free diet. Refeeding the same diet shows the response of rats to fasting-refeeding alone, and refeeding the fat-free diet illustrates how a lack of dietary linoleate affects the fasting-refeeding responses.

EXPERIMENTAL PROCEDURES

Male Sprague-Dawley rats (Simonsen Farms, Gilroy, CA) were caged individually in suspended, galvanized, wire-bottom cages. The diets and tap water were supplied ad libitum.

In the first experiment, 150 g-male rats were fed the purified casein-sucrose diet (14) supplying 5% safflower oil for 3 wk. They were then divided into two groups, similar in body weight, and fasted for 48 hr. One group was then refed the 5% safflower oil diet ad libitum. The other group was fed the same diet with the safflower oil omitted. Body weights and food intakes were recorded daily. The rats were sacrificed after 3 and 7 days of refeeding.

In the second experiment, weanling rats were fed the 5% safflower oil diet for 7 wk, then fasted for 48 hr, and refed the fat-free diet ad libitum for 1, 2, 3, and 7 days. Body weights and food intakes were recorded daily.

The rats were sacrificed by decapitation. Livers were removed, weighed, lyophilized, and extracted for lipid analyses as previously described (14). Glucose-6-phosphate dehydrogenase (G6PD) (D-glucose-6-phosphate; NADP oxido-reductase, EC 1.1.1.49) and fatty acid synthetase (FAS) were measured as described (9). Statistical significance of differences between means was calculated by the Student "t" test (15).

RESULTS

Experiment 1

Body weights were similar in rats refed either the 5% safflower oil diet or the fat-free diet for 3 or 7 days (Table I). Food intake by rats refed the fat-free diet was slightly greater, but energy intake was slightly less than that of rats refed the 5% safflower oil diet. These data on energy intake do not agree with the conclusions of Gozukhara et al. (16) that the presence of dietary fat reduces the energy intake of rats refed after fasting.

In rats refed the 5% safflower oil diet for 3 days, G6PD activity was about three times as high as the nonfasted level. In rats refed the fat-free diet for 3 days, enzyme activity reached almost five times the nonfasted level. After 7 days refeeding the safflower oil diet, G6PD activity decreased to about twice the nonfasted level, but after refeeding the fat-free diet for 7 days, G6PD activity remained at four times the nonfasted level. The activities at 3 and 7 days in the rats refed the fat-free diet did not differ significantly ($P > 0.1$).

Data for liver total fatty acids are given in Table II. In rats refed the safflower oil diet for 3 days, the *proportion* of arachidonate in total fatty acids was much lower than the fasting value, although the *amount* (mg) of arachidonate per liver was greater in the refed rats. After 7 days, the proportion of arachidonate was twice as high as after 3 days refeeding, even though mg arachidonate per liver showed only a small increase. The proportion of linoleate decreased after 3 days refeeding and increased after 7 days.

In rats refed the fat-free diet, arachidonate in total fatty acids decreased to 2.6% after 3 days refeeding and increased to 7.2% after 7 days. The amount of arachidonate per liver also increased between 3 and 7 days. Linoleate decreased to 2% after 3 days refeeding, with little change after 7 days. Eicosatrienoate increased to about 1%.

With either diet, total fatty acids (mg/g liver or mg/liver) more than doubled after 3 days refeeding. These levels decreased considerably after 7 days, to values about 50% greater than the nonfasted values.

TABLE I

Effects of Fasting and Refeeding on Body Weight, Food Intake, and Liver Glucose-6-phosphate Dehydrogenase Activities in Rats. (Rats were fed a 5% safflower oil diet before fasting and were refed either the same diet or a fat-free diet.)

Dietary treatment[b]	Body weight				Cumulative 3-day intake			Cumulative 7-day intake			G6PD Activity[a]			
			Refed										Refed	
	Nonfasted	Fasted	+3 days	+7 days	Food	Sucrose	Kcal	Food	Sucrose	Kcal	Non fasted	Fasted	+3 days	+7 days
			g		g	g		g	g					
5%-5%	252 ±2[c] (16)[d]	238 ±2 (16)	276 ±3 (16)	299 ±5 (8)	63.8 ±2.6 (16)	40.6 ±3.8 (16)	262	141.6 ±4.5 (8)	95.4 ±3.0 (8)	582	85 ±15 (6)	58 ±7 (12)	268 ±22 (8)	163 ±17 (8)
5%-0	253 ±2 (16)	240 ±2 (16)	275 ±2 (16)	302 ±3 (8)	65.0 ±1.4 (16)	47.2 ±1.0 (16)	251	147.3 ±2.4 (8)	106.9 ±1.8 (8)	568			406 ±38 (8)	360 ±46 (8)

[a]G6PD Activity: nmoles NADP+ reduced per min per mg protein.

[b]Rats were fed the 5% safflower oil diet (5%-5%) or the 0 fat diet (5%-0) for 3 and 7 days after fasting for 48 hr.

[c]Mean and standard error.

[d]Number in parentheses is the number of rats per group.

TABLE II

Total Fatty Acids in Livers of Rats Refed either a 5% Safflower Oil Diet or a Fat-free Diet after a 48-hr Fast. (All rats were fed the 5% safflower oil diet before fasting.)

Dietary treatment[a]	Liver weight	Total fatty acids	Liver total fatty acids[b]							Liver arachidonate	
			16:0	16:1	18:0	18:1	18:2	20:3	20:4		
	g	mg/g	Percentages by weight							mg/g	mg/liver
5% nonfasted	11.3 b 0.8	36 ± 3	22.6	4.0	14.1	16.3	15.7	–	18.6	6.7 ± 0.3	75 ± 5
5% fasted 48 hr	7.1 ± 0.6	33 ± 1	18.8	1.9	17.1	9.3	22.4	–	23.0	7.6 ± 0.2	54 ± 5
5%-5%, refed 3 days	13.4 ± 0.3	84 ± 9	31.9	9.7	7.6	30.9	6.6	–	6.7	5.6 ± 0.3	75 ± 2
5%-5%, refed 7 days	12.7 ± 0.5	49 ± 6	29.0	6.5	11.4	23.3	10.8	–	13.0	6.4 ± 0.4	81 ± 3
5%-0, refed 3 days	13.2 ± 0.4	96 ± 13	35.6	12.0	5.6	37.5	1.6	0.6	2.6	2.5 ± 0.7	35 ± 10
5%-0, refed 7 days	14.0 ± 0.1	52 ± 1	29.0	8.7	12.2	31.5	2.8	1.3	7.2	3.7 ± 0.6	52 ± 9
Pooled S.E.			1.0	0.6	0.8	1.5	0.7	0.1	1.0		

[a]Mean and standard error, 4 rats per group.

[b]The values for 14:0, 22:5, and 22:6 have been omitted from the table. The proportion of 14:0 was less than 2% in all groups. The proportions of 22:5 and 22:6 together did not exceed 4% in any of the groups.

TABLE III

Fatty Acids in Liver Phospholipids in Rats Refed either a 5% Safflower Oil Diet or a Fat-free Diet after a 48-hr fast. (All rats were fed the 5% safflower oil diet before fasting.)

	Liver phospholipid fatty acids Percentages by weight									
Dietary treatment[a]	14:0	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:5	22:6
5% nonfasted	0.3	19.0	1.8	20.7	6.8	13.1	–	29.1	–	2.4
5% fasted 48 hr	0.2	18.8	1.0	23.0	5.2	14.2	–	32.0	1.2	2.6
5%-5%, refed 3 days	0.4	19.4	2.2	22.0	8.6	9.4	–	29.0	0.8	0.5
5%-5%, refed 7 days	0.4	20.4	2.0	20.9	7.1	12.0	–	28.7	1.4	0.7
5%-0, refed 3 days	0.6	24.0	3.5	18.6	21.0	5.8	3.8	18.4	2.9	2.1
5%-0, refed 7 days	0.5	22.1	4.8	19.9	16.5	5.2	6.0	19.8	3.2	2.3
Pooled S.E.	0.1	0.9	0.3	0.8	0.5	0.4	0.9	0.9	0.6	0.2

[a]Mean. Four rats per group.

TABLE IV

Body Weights and Activities of Glucose-6-phosphate Dehydrogenase and Fatty Acid Synthetase in Livers of Rats Refed a Fat-free Diet after a 48-hr Fast. (All rats were fed a 5% safflower oil diet before fasting.)[a]

		Days of refeeding			
Nonfasted	Fasted	+1	+2	+3	+7
		Body weight, g			
298 ± 3	272 ± 2	288 ± 3	295 ± 3	298 ± 3	315 ±10
		Glucose-6-Phosphate dehydrogenase (nmoles NADP reduced x min^{-1} x mg $protein^{-1}$)			
86 ±15	58 ± 6	146 ±19	292 ±22	398 ±14	552 ±10
		Fatty acid synthetase (nmoles NADPH oxidized x min^{-1} x mg $protein^{-1}$)			
47 ± 7	15 ± 2	61 ±17	106 ± 5	117 ±11	116 ± 1

[a]Mean and standard error. Three rats per group.

Fatty acids of liver phospholipids are shown in Table III. Fasting produced little change in the proportions of fatty acids in liver phospholipids. Refeeding the 5% safflower oil diet also had little influence on percentage distributions of fatty acids in this fraction. Arachidonate remained at ca. 29%. Linoleate decreased only from 13% to 9% after 3 days and returned to 12% after 7 days. The profound changes seen in liver total fatty acids (Table II) must have been caused, therefore, by changes in other lipid fractions, probably triglycerides. The increases in triglycerides are shown by the increases in total fatty acids (Table I).

In contrast, upon refeeding the fat-free diet, phospholipid arachidonate decreased to 18.4% and linoleate to 5.8% after 3 days, and these levels were maintained after 7 days. Eicosatrienoate increased to 6% after 7 days.

Experiment 2

To compare EFA depletion and enzyme activities immediately after refeeding the fat-free diet as well as after 7 days, the rats were adapted to the 5% safflower oil diet, fasted for 48 hr, and then refed the fat-free diet for 1, 2, 3, and 7 days. The rats regained their prefasting weight in 2 days and continued to gain (Table IV). Activity of G6PD was approximately twice the nonfasted level after one day of refeeding and continued to rise up to 7 days. Activity of fatty acid synthetase (FAS) after one day of refeeding was slightly higher than in nonfasted rats and was about twice as high after 2 days' refeeding. FAS activity after 3 and 7 days refeeding ramined at about 2.5 times the nonfasted level.

Refeeding produced a large increase in total fatty acids. The values increased to 99 mg/g liver in the 3-day refed rats and decreased to 79

TABLE V

Changes in the Proportions of Arachidonate, Linoleate, and Eicosatrienoate in Liver Total Fatty Acids and in Phospholipid Fatty Acids during Refeeding of a Fat-free Diet after a 48-hr Fast. (All rats were fed the 5% safflower oil diet before fasting.)[a]

Liver total fatty acids Percentages by weight						Liver phospholipid fatty acids Percentages by weight					
		Day of refeeding						Day of refeeding			
Nonfasted	Fasted	+1	+2	+3	+7	Nonfasted	Fasted	+1	+2	+3	+7
					Arachidonate						
20.4 ±1.8	25.5 ±1.8	14.7 ±1.3	6.3 ±0.7	3.7 ±1.5	3.9 ±0.8	30.7 ±0.6	33.9 ±0.2	25.2 ±0.3	20.5 ±0.6	19.4 ±1.8	20.4 ±0.8
					Linoleate						
15.7 ±1.2	17.9 ±0.6	7.7 ±0.6	2.5 ±0.3	1.6 ±0.4	1.5 ±0.2	13.4 ±0.8	13.5 ±0.6	9.6 ±0.4	6.4 ±0.5	5.8 ±0.1	5.1 ±0.2
					Eicosatrienoate						
–	0.1 ±0.1	0.4 ±0.1	0.9 ±0.2	0.9 ±0.2	1.6 ±0.5	–	–	0.6 ±0.1	3.0 ±0.3	4.9 ±0.3	7.6 ±1.0

[a]Mean and standard error. Three rats per group.

mg/g after 7 days. Arachidonate (mg/g liver) decreased from 5.4 in the nonfasted rats to 2.6 after 3 days refeeding, in good agreement with corresponding values in the first experiment.

Table V shows the effects of refeeding a fat-free diet on the proportions of fatty acids in liver total fatty acids and in phospholipid fatty acids. As expected from the first experiment, refeeding produced sharp declines in percentages of arachidonate and linoleate in total fatty acids and caused eicosatrienoate to appear. In contrast, liver phospholipids showed no changes in percentages of arachidonate or linoleate with fasting and only a relatively slow loss of arachidonate and linoleate during refeeding. Arachidonate in phospholipid fatty acids was 30.7% before fasting and 20.5% after 2 days refeeding, with no additional decreases after 3 and 7 days (Table V). Eicosatrienoate increased to 4.9% and 7.6% after 3 and 7 days refeeding. Linoleate was 13.4% in nonfasted rats and decreased to 9.6% and 6.4% after one and two days refeeding.

DISCUSSION

In fasting-refeeding studies, it is important to distinguish between two different phenomena: responses to refeeding alone and responses to changes in composition of the diet used in refeeding. To study the first phenomenon, animals should be refed a diet of the same composition as that eaten before fasting. In this way, responses to fasting can be observed without complications from changes in dietary composition. When effects of dietary ingredients are sought, the diet used for refeeding should differ in only one component from the prefasting diet. When chow-fed rats are fasted and refed a purified diet, their responses will be an inscrutable mixture of those caused by fasting-refeeding and those caused by the multitudinous changes in food composition.

Our results with rats fed the 5% safflower oil diet before and after fasting thus represent effects due to fasting-refeeding alone, without dietary change. Under these conditions, the proportion of arachidonate in liver phospholipids and the mg arachidonate per liver were maintained at the prefasting levels. Liver G6PD activity increased to three times the prefasting level after 3 days refeeding but after 7 days declined to only twice the prefasting level. In rats refed tha fat-free diet after previous adaptation to the 5% safflower oil diet, the changes are the combined responses to removal of dietary EFA as well as to fasting-refeeding. Since safflower oil contains more than 70% linoleate, the removal of safflower oil represents chiefly removal of EFA. In this situation, the proportion of arachidonate in phospholipids and the mg arachidonate per liver decreased, in contrase to the results with the rats refed the 5% safflower oil diet. The values for G6PD in rats refed the fat-free diet were five times the prefasting value after 3 days refeeding and four to six times the prefasting level after 7 days refeeding. The activity of fatty acid synthetase was twice the prefasting level after 7 days refeeding. Wiegand et al. (17) reported a similar elevation of FAS activity in chow-fed rats fasted and refed a fat-free diet for 6 days.

Recent work (18,19) indicates that an in-

active form of G6PD is produced in rats and mice fed diets high in fat. Removal of dietary fat increases the amount of active enzyme without altering its rate of synthesis or degradation (18). Such a phenomenon could explain the persistence of elevated G6PD activities after 7 days of refeeding the fat-free diet in our experiments, as well as the decline in G6PD activity in rats refed the 5% safflower oil diet. Palmitoyl CoA inhibition of yeast G6PD has been reported (20). However, these observations (18-20) do not explain why EFA are more effective than equal amounts of nonessential fatty acids in reducing activity of liver G6PD and other lipogenic enzymes in rat liver (6,9,21). Furthermore, our results with rats refed the 5% safflower oil diet show that maintenance of phospholipid arachidonate during refeeding does not prevent the initial increase in G6PD with refeeding. The increased activity produced by refeeding does decline if the diet supplies EFA but remains elevated if the liver becomes depleted of EFA, i.e. when an EFA-deficient diet is refed. This is consistent with the evidence that increased activities of liver lipogenic enzymes occur in EFA-deficient rats fed ad libitum (8,9.14).

Severe EFA depletion has been inferred from the decreased proportion of arachidonate in liver total fatty acids of rats refed a fat-free diet (1). This has led to the suggestion that fasting-refeeding was a rapid means of producing EFA-depletion in adult rats. Our data show clearly that expression of arachidonate only as a *proportion* of total liver fatty acids is very misleading. In refed rats, the large decrease in the proportion of arachidonate in liver total fatty acids occurred, not because of a large decrease in the amount of arachidonate, but rather because of large increases in other liver fatty acids after refeeding (22,23) especially in palmitate and oleate in triglycerides.

The increases in eicosatrienoate in rats refed the fat-free diet show, of course, that EFA depletion does occur in the liver under these conditions, although the depletion was much less severe than in rats fed an EFA-deficient diet for 4 wk post-weaning (14). The availability of linoleate from body stores (24) is indicated by maintenance of relatively constant proportions of linoleate (5-6%) and arachidonate (18-20%) in liver phospholipids from the third to the seventh day of refeeding. Yet the amount of EFA available from nonhepatic tissues is not enough to restore prefasting linoleate and arachidonate levels in the liver.

Because of the importance of endoplasmic reticulum in lipogenesis and desaturation of fatty acids, Park and coworkers (13) investigated changes in the fatty acid composition of phospholipids of rough and smooth microsomes during fasting-refeeding. In rats fed a chow diet before fasting and refed a 5% corn oil diet for 2 days, more EFA were lost from the phosphatidylcholines and phosphatidylethanolamines of rough microsomes than from these phospholipids in either smooth microsomes or total liver. Park et al. suggested that the decreases in EFA of rough microsomes might result from lipoprotein secretion and might be related also to the increased activity of liver lipogenic enzymes in fasted-refed rats (1,2,6,9).

The work of Park et al. is very valuable for showing how fasting affects lipid composition of cell organelles associated with lipogenesis and fatty acid desaturation. Yet, these observations do not separate the effects of fasting-refeeding from the combined effects of fasting-refeeding and change of diet composition. In addition, differences were noted in liver lipid composition between nonfasted rats fed the chow diet and nonfasted rats fed the purified diet. Consequently, the results of Park and coworkers need to be confirmed by a similar study with rats refed the high carbohydrate diet before as well as after fasting in order to assess validly the fatty acid changes produced by fasting-refeeding.

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Pancreatic and Microbial Lipases: A Comparison of the Interaction of Pancreatic Colipase with Lipases of Various Origins

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ABSTRACT

Conjugated bile salts inhibit the hydrolysis of triglycerides (TG) by the lipases from *Rhizopus arrhizus* and *Geotrichum candidum*. This occurs for detergent concentrations similar to those which suppress the action of mammalian pancreatic lipases upon the same substrates. However, in opposition with what is observed with the latter enzymes, the activity is not restored by the addition of pancreatic colipase. Both pancreatic and *R. arrhizus* lipases are inactivated at tributyrin/water interface, but only the first enzyme is protected against this surface denaturation by the pancreatic cofactor. These observations suggest that colipases synthesized in mammalian pancreas display specific interaction towards the lipases made by the same organ.

INTRODUCTION

Lipases (glycerol ester hydrolases, EC 3.1.1.3) are widely distributed in animals, plants, and microorganisms (1). Extensive studies on the mode of action of pancreatic lipase from higher animals have demonstrated the unique interaction which exists between enzyme and substrate molecules at the lipid/water interface (2,3). Kinetic studies of the lipase catalyzed hydrolysis of short and long chain triglycerides show that, in the absence of a protein cofactor (colipase), conjugated bile salts inhibit the interfacial reaction. In the presence of colipase, pancreatic lipase is fully active even at bile salt concentration well above the critical micelle concentration (4-6). Colipase has been isolated from pig and beef pancreas. The porcine cofactor is a small protein (mol wt about 10,000 daltons). Its primary structure was elucidated by Charles et al. (7). It must be noted that the inhibitory effect of bile salt upon the activity of a given mammalian pancreatic lipase can be suppressed by pancreatic colipase coming from other mammals (5,8).

The mechanism of this inhibition and of its reversal by a protein cofactor has been much discussed. There is evidence that, once conjugated bile salts are covering a lipid/water interface, pancreatic lipase can adsorb onto it only if sufficient cofactor is present (i.e. lipase and colipase are in equimolar amounts).

In vitro studies have demonstrated that one molecule of colipase interacts with one bile salt micelle. This complex can associate to one molecule of enzyme (9). Sari et al. (10) have shown that the formation of the binary complex induces a strong perturbation of the ultraviolet spectrum of the tyrosines of colipase. This spectral perturbation might reflect a conformational change of the corprotein that creates a specific site of recognition for the enzyme. In line with this hypothesis, Chapus et al. have proposed a model accounting for colipase activation of bile salt inhibited pancreatic lipase (11). In recent studies on the effect of colipase and taurodeoxycholate on porcine pancreatic lipase activity, Momsen and Brockman (12,13) came to the general conclusion that bile salt inhibition results from the interaction of the enzyme with detergent molecules which takes place below the critical micelle concentration of bile salt. They demonstrated that colipase helps to stabilize lipase at the lipid/water interface by preventing surface inactivation of the enzyme and they were able to confirm that colipase can form a highly stable 1:1 complex with lipase-bile salt.

In order to better characterize the specificity of interaction of colipase with lipase, we investigated the effect of the pancreatic coprotein on the activity of lipases of different origins. Lipases from the molds *Rhizopus arrhizus* and *Geotrichum candidum* were selected for this study. These enzymes are now available in pure form and some of their properties are well characterized (14,15). As in vertebrate lipases, *Rhizopus* lipase shows a high specificity for the external positions of long chain triglycerides while *G. candidum* lipase, which is inactive towards short chain triglycerides such as tributyrin, has unique catalytic properties towards unsaturated fatty acids. Results reported here indicate that both pancreatic and mold enzymes are inhibited at the lipid/water interface by addition of bile salt or by surface denaturation. Colipase activates and stabilizes

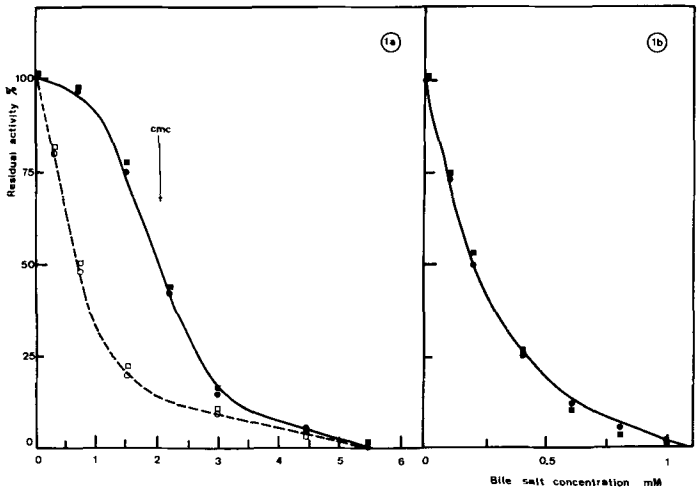

FIG. 1. Effect of the bile salt concentration on the initial rate of hydrolysis of tributyrin and triolein by microbial lipases in the absence and in the presence of an excess amount of pancreatic colipase. Hydrolysis of emulsified tributyrin and triolein was determined at 25 C and at pH 8.0 and 9.0, respectively. Mixed bovine bile salts and sodium taurodeoxycholate have similar effect on enzyme hydrolysis. FIG. 1a. Hydrolysis of triolein by *Rhizopus* lipase (full line) and by *G. candidum* lipase (dotted line) in the absence (circles) and in the presence (squares) of bovine pancreatic colipase. FIG 1b. Hydrolysis of tributyrin by *Rhizopus* lipase.

pancreatic lipase but has no effect on the microbial enzymes.

MATERIALS AND METHODS

Sheep pancreatic lipase (mol wt: 49,000 daltons), free of colipase, and bovine colipase A were prepared in pure form in our laboratory according to previously described method (16,17). *R. arrhizus* lipase (mol wt: 40,000 daltons) was purchased from PRECIBIO, Paris, France. *G. candidum* lipase was obtained from M. Iwai, Osaka, Japan. Mixed bovine bile salts (critical micelle concentration: 2 mM) were prepared from bile and pure sodium taurodeoxycholate was purchased from Sigma (St. Louis, MO). Lipase activity against tributyrin and triolein was determined potentiometrically at pH 8.0 and 9.0, respectively. Experimental procedures for activity determination have been described in detail in previous reports (8,18). In all assays, the substrate concentration allowed the reaction to proceed at its maximum rate. Inhibition studies were carried out in the presence of an amount of enzyme corresponding to about five enzyme units. One lipase unit corresponds to the liberation of one microequivalent fatty acid per minute in standard conditions. The actual concentration of enzyme in kinetic studies (see Fig. 2) is $3.5.10^{-9}$M for *Rhizopus* lipase and $9.5.10^{-10}$M for pancreatic lipase. Activation experiments were performed in the present of a large excess of colipase (30 to 50 colipase units). One colipase unit is the amount of cofactor that increases bile salt inhibited pancreatic lipase activity by one enzyme unit (8). Protein concentration was determined by the Lowry colorimetric method.

RESULTS AND DISCUSSION

Rhizopus and *G. candidum* lipases were first assayed against gum arabic emulsion of triolein in the presence of an increasing concentration of bile salt. Two series of assays were performed in the absence and in the presence of an excess amount of pancreatic colipase. Bile salt activity dependence curves are shown in Figure 1a.

The curves show that microbial lipases are inhibited by bile salt in the same concentration range as mammalian pancreatic lipases (8). However, colipase does not restore enzyme activity. Experiments carried out with *Rhizopus* lipase acting on emulsified tributyrin give very similar results (Fig. 1b). Inhibition of short chain triglyceride hydrolysis occurs at bile salt concentration below the critical micelle concentration, as found with pancreatic lipase (18) and colipase, again, has no activating effect either in the absence or in the presence of bile salt. Although pancreatic and microbial lipases seem to be equally sensitive to bile salt, most probably due to a general mechanism of inhibition, they are different with respect to their ability to interact with the pancreatic

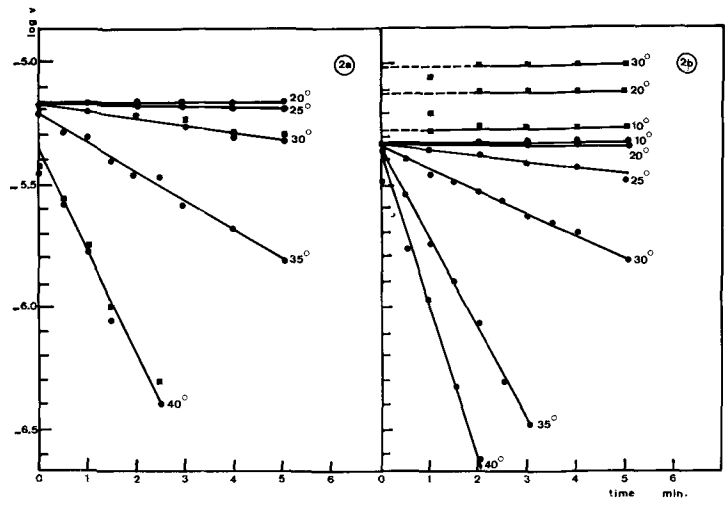

FIG. 2. Relation of lipase activity against emulsified tributyrin with reaction time. Kinetics were studied at pH 8.0 in the absence (circles) and in the presence (squares) of an excess amount of colipase. FIG. 2a. Assays with *Rhizopus* lipase. FIG. 2b. Assays with sheep pancreatic lipase (the dotted line represents the presteady state of the reaction corresponding to the lipase binding to the surface).

cofactor. In these experiments, colipase behaves as a specific activator for mammalian pancreatic lipases.

Further evidence that pancreatic colipase fails to interact with microbial lipases at the substrate/water interface was obtained from kinetic experiments. The kinetics of the hydrolysis reaction of emulsified tributyrin by sheep pancreatic lipase were studied at pH 8.0 and at temperature ranging from 10 C to 40 C, in the absence and in the presence of colipase. Parallel experiments were carried out with the *Rhizopus* enzyme.

The velocity of the reaction (v) was calculated from the kinetics, at different times (t) of the reaction, and log v was plotted against t (Fig. 2).

At temperatures above 20 C and in the absence of colipase, the velocity of the reaction decreases with time for both enzymes. At these temperatures, the reaction follows first order kinetics up to 80% of maximum hydrolysis as previously found by Momsen and Brockman in kinetics studies of porcine pancreatic lipase catalyzed hydrolysis of tripropionin dispersed in water, in the presence of glass beads (12). According to their interpretation, the observed decrease of the reaction rate results from irreversible inactivation of the enzyme at the interface. Assuming that at zero time all the lipase molecules are in an active form at the lipid/water interface and further become inactive upon surface denaturation according to a first-order reaction:

$$E_o = E_a + E_d$$

E_o = total concentration of lipase at the interface, E_a = concentration of active molecules of lipase, E_d = concentration of denatured molecules of lipase, then the rate of inactivation is:

$$-\frac{dE_a}{dt} = \frac{dE_d}{dt} = k_d E_a$$

with k_d, denaturation constant of lipase at the interface. Integration yields:

$$E_a = E_o e^{-k_d t}$$

and the rate of hydrolysis is:

$$v = k_{cat} E_a = k_{cat} E_o e^{-k_d t}$$

The plot of log v versus t gives straight lines with slopes representing $-k_d/2.3$. The intercept at time zero is log $k_{cat}E_o$. Values of k_d for *Rhizopus* and pancreatic lipases at temperatures between 25 C and 40 C are given in Table I.

Values of Table I indicate that under these conditions, surface denaturation is quite comparable for both enzymes.

When assays were performed with colipase, values of k_d for *Rhizopus* lipase remained unchanged, whereas the kinetics of tributyrin hydrolysis by the pancreatic enzyme were strongly affected. It can be noticed, first, that sheep lipase activity is maximal only after 1 to 2 min as observed in previous studies (13,19). This lag period corresponds to the adsorption of lipase to the substrate surface. The velocity of hydrolysis then remains constant within the first 5 min of the reaction. It is thus clear that

TABLE I

Effect of Temperature on k_d (sec^{-1}), the First Order Inactivation Constant of Sheep Pancreatic Lipase and *Rhizopus arrhizus* Lipase at the Tributyrin/Water Interface in the Absence of Colipase and Bile Salt

Temperature	25 C	30 C	35 C	40 C
Pancreatic lipase	$1.1\ 10^{-3}$	$3.2\ 10^{-3}$	$13.8\ 10^{-3}$	$23.0\ 10^{-3}$
Rhizopus lipase	$0.1\ 10^{-3}$	$1.1\ 10^{-3}$	$11.2\ 10^{-3}$	$15.4\ 10^{-3}$

colipase, by interacting with lipase, stabilizes the enzyme at the tributyrin/water interface and protects it against surface denaturation. Moreover, in contrast to what is observed without colipase, the pancreatic lipase catalyzed reaction becomes temperature-dependent and follows the classical Arrhenius equation. The energy of activation of tributyrin lipolysis calculated from the maximum activity at temperatures ranging from 10 C to 40 C is 5.25 Kcal $mole^{-1}$. From these observations, it might be hypothesized that, in the absence of colipase, the overall reaction of hydrolysis is controlled by a rate-limiting step which is not temperature-dependent. However, this assumption is in opposition with the fact that the hydrolysis of tributyrin between 0 C and 10 C is temperature-dependent in the absence of colipase (not shown). The energy of activation (5 Kcal $mole^{-1}$) is similar to that found at temperatures above 10 C, in the presence of colipase. Therefore, it can be concluded that colipase has no direct effect on the k_{cat} of the hydrolysis reaction but, more likely, helps to keep the enzyme in an active conformation at the lipid/water interface. Some time ago, it was shown by Esposito et al (20) that the activity of porcine pancreatic lipase against ester monolayers was strongly dependent upon the surface pressure of the film. The assumption was made that when the free interfacial energy differs from the optimal value for enzyme activity only part of the adsorbed enzyme is in a functional conformation. In line with this hypothesis, it can be suggested that lipase adsorbed at the tributyrin/water interface exists in two forms, an active and an inactive form, which are in equilibrium:

$$E_{ads}\ (\text{active}) \rightleftharpoons E_{ads}\ (\text{inactive})$$

In the absence of colipase, an increase in temperature would have two antagonistic effects. First, it would decrease the concentration of the active form of adsorbed lipase and second, it would increase the value of k_{cat} in such a way that the observed rate of lipolysis remains constant. With colipase, all pancreatic lipase molecules at the interface would be in the active form in the range between 10 C and 40 C and the increase of the velocity of the hydrolysis reaction would result only from the effect of temperature on k_{cat}. Then, if one assumes that only lipase molecules in the inactive form are sensitive to irreversible surface denaturation,

$$E_{ads}\ (\text{active}) \rightleftharpoons E_{ads}\ (\text{inactive}) \overset{k_d}{\rightarrow} E_d$$

the formation of a colipase-lipase complex would be critical to the stabilization of all enzyme molecules in their active conformation and would make the lipolysis reaction temperature-dependent. This hypothesis can help to interpret the activating effect of colipase on tributyrin hydrolysis by pancreatic lipase in systems containing no bile salt. As observed by several investigators (6,21,22), addition of colipase produces a twofold increase of the initial rate of hydrolysis at 20 C. This activation might be due merely to the presence at the lipid/water interface of all lipase molecules in the form of the active cofactor-enzyme complex.

It is known, since the original studies of Olive and Dervichian (23) on the hydrolysis of ester monolayers by *Rhizopus* and pancreatic lipases, that the activity of both enzymes strongly depends upon the surface pressure of the film. It is thus reasonable to conclude that the failure of colipase to stabilize *Rhizopus* lipase at the tributyrin/water interface is a good evidence that actually no stable complex between the coprotein and the *Rhizopus* enzyme is being formed. Further investigations on the interaction of pancreatic colipase with other lipases will help to characterize better the specificity of the cross interaction of the two proteins in particular amongst the animal phyla. A phylogenic study of this specific interaction might, indeed, contribute to a better understanding of the physiological role of the protein cofactor.

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Fatty Acids and Sterols of *Cronartium fusiforme* Basidiospores

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ABSTRACT

The hydrocarbons, fatty acids, and sterols of *Cronartium fusiforme* basidiospores were examined by gas chromatography and mass spectrometry. Trace quantities of aliphatic hydrocarbons were detected, but these were probably not fungal products. Principal nonsubstituted fatty acids were palmitic (11.7%), linoleic (17.2%), and linolenic (16.1%) acids; the predominant acid was 9,10-*cis*-epoxyoctadecanoic acid (40.7%). Four sterols were detected, three of which were identified as stigmast-7-enol (57.6%), stigmasta-5,7-dienol (22.7%), and ergost-7-enol (16.0%). The fourth sterol (4%) is a C_{28} diene. Lipids of the aeciospores and basidiospores of *C. fusiforme* are compared.

INTRODUCTION

Chemical analyses of rust fungi have been restricted to the various spore types (uredospores, teliospores and aeciospores) because, until recently, rusts could not be cultured in the laboratory. Although they can now be cultured, axenic cultures of rust fungi show very sparse growth and do not produce spores characteristic of those that occur in the natural life cycle. In nature, frequently one spore type is produced abundantly, or at least in sufficient quantity to be collected for study, whereas the other spore forms of that species are produced in relatively limited amounts. This is the case with *Cronartium fusiforme* Hedge and Hunt ex Cumm., which causes fusiform rust of pines in the southern United States. Large numbers of aeciospores are produced in galls on the pine host each spring; its other spore forms are produced in only limited amounts, making it difficult or impossible to collect them from naturally infected materials in quantities sufficient for analysis. However, as part of a program for screening pine trees for resistance to fusiform rust, USDA Forest Service personnel, Forest Resource Protection in Ashville, NC are obtaining *C. fusiforme* basidiospores from an oak alternate host (*Quercus rubra*) artificially inoculated with aeciospores. Small quantities of basidiospores were made available to us and this note reports their fatty acid and sterol composition. To our knowledge, this is the first report of the lipid composition of basidiospores of a rust fungus.

EXPERIMENTAL PROCEDURES

Basidiospores were collected by suspending oak leaves with germinating teliospores over acidified water in petri dishes. Liberated basidiospores were concentrated by filtration on millipore filter pads and stored at about 5 C. Before shipping, the spores were frozen and packed in dry ice. Upon arrival, the spores were thawed, washed from the filter pad with distilled water, concentrated by low-speed centrifugation, refrozen, and dried by lyophilization. Dry basidiospores, 0.2 to 0.4 g, were weighed and then ground in a mortar with an equal amount of neutral alumina. The ground mixture was then added to 150 ml chloroform-methanol (2:1, v/v), warmed briefly, and then stirred magnetically for 2 hr. The cell fragments and alumina were removed by centrifugation and washed with 50 ml chloroform. Cell fragments and alumina were removed as before and the extract plus wash was concentrated under nitrogen. The extraction was considered complete since virtually all of the carotenoid pigment was removed from the spores.

The extract was analyzed for hydrocarbons, fatty acids, and sterols. A portion of the extract was placed on a 6 cm x 2 cm silica gel (60-200 mesh) column and the hydrocarbons were eluted with petroleum ether. All solvents used in this study were of nanograde quality (Mallinkrodt, Inc.). Total fatty acids were analyzed as their methyl esters, which were prepared by transesterification of the total lipid extract using 0.5 N sodium methoxide in methanol (Applied Science Laboratories, State College, PA). Free sterols were isolated from the total lipid by digitonin precipitation. One drop of 10% acetic acid and 1.5 ml of 0.5% digitonin were added to the total lipid fraction in 3 ml acetone-ethanol (1:1, v/v) and the mixture was stirred on a Vortex mixer prior to storing overnight at room temperature. The digitonide was collected by low-speed centrifugation and washed first with acetone-diethylether (1:2, v/v) and then two times with diethylether. Free sterols were recovered by dissolving the digitonides in 10 ml dimethyl sulfoxide in a 40 ml centrifuge tube which was

placed in boiling water bath for 1 hr (1). The solution was cooled to room temperature and washed three times with 5 ml of petroleum ether. The combined washes which contained the free sterols were taken to dryness under nitrogen. Sterols were analyzed as free alcohols and as acetate and trimethylsilyl ether (TMS) derivatives. Acetate derivatives were prepared by dissolving the free sterols in pyridine-acetic anhydride (1:1, v/v) and warming for 10 min. TMS derivatives were prepared with Tri-Sil (Applied Science Laboratories).

Epicuticular wax of oak leaves was also examined. Wax was removed by dipping the leaves in chloroform for 15 sec (2). The chloroform was evaporated under nitrogen and the hydrocarbons were isolated by silica gel column chromatography as described above.

Hydrocarbons, fatty acids, and sterols were analyzed by gas chromatography (GLC) using either a Varian Aerograph 2440 or 1400 gas chromatograph equipped with flame ionization detectors. Hydrocarbons and sterols were separated on a 3 m x 2 mm ID glass column packed with 3% SE-30 on Chromosorb Q. Column temperature for hydrocarbon anaylses was programmed from 150 to 270 C at 4 C/min; for sterol analyses the temperature was 250 or 270 C isothermal. Fatty acids were separated on a 3 m x 2 mm ID stainless steel column packed with 12% DEGS (diethylene glycol succinate) on Gas Chrom P. Column temperature for fatty acids analyses was 180 C isothermal and the injector and detector temperatures for all GLC analyses were 270 C. Identification of individual lipids was made by comparison of GLC retention times with those of authentic standards and/or by GLC-mass spectrometry. Mass spectrometric (MS) analyses were made on a DuPont 21-490 single-focusing mass spectrometer linked to a Varian Aerograph 1400 gas chromatograph. All MS analyses were conducted with the source temperature at 200 C and an ionizing voltage of 70 eV.

RESULTS AND DISCUSSION

The total lipid content of *C. fusiforme* basidiospores was ca. 31%, which is high compared to most fungal spores (3). Rust spores generally have a high lipid content, ranging between 4 and 20% of the spore weight. Aeciospores of *C. harknessii, C. ribicola,* and *C. commandrae* contained 12.5, 18, and 17% (4,5) total lipid, respectively, while *C. fusiforme* aeciospores contained 3.7% (6).

Rust and smut spores generally contain a homologous series of n-alkanes ranging in chain-lengths from C_{16} to C_{36}, with C_{27}, C_{29}, and C_{31} predominating (3,7). Basidiospores of *C. fusiforme* contained only trace quantities of aliphatic hydrocarbons ranging in chain-length from about C_{21} to C_{36}. However, there was no odd over even carbon chain predominance characteristic of hydrocarbons from fungal and higher plant materials (7,8). The typical alkane pattern was not observed with *C. fusiforme* aeciospores (6). Since it was not possible to see that precautions against contamination by petroleum products were taken during spore collection, we cannot be certain that the hydrocarbons detected were fungal products. Although the hydrocarbon pattern of the basidiospores was not typical of higher plants, alkanes from the host epicuticular wax were determined. The oak hydrocarbon pattern was not similar to that of the basidiospores, but was typical of higher plant epicuticular wax (8). Alkanes ranged from C_{23} to C_{33}, with C_{27}, C_{29} and C_{31} being the predominant homologues.

TABLE I

Fatty Acids and Sterols of *C. fusiforme* Basidiospores

Fatty acids	%	Sterols	%
C_{14}	1.2	stigmast-7-enol	57.6
C_{15}	0.3		
C_{16}	11.7	stigmasta-5,7-dienol	22.7
$C_{16:1}$	0.7		
C_{17}	1.3	ergost-7-enol	16.0
C_{18}	3.2		
$C_{18:1}$	3.5	C_{28} diene	4.0
$C_{18:2}$	17.2		
C_{20}	4.1		
$C_{18:3}$	16.1		
epoxy C_{18}	40.7		

Fatty acids of rust spores are generally similar to those of other fungi and of higher plants (3); however, rust fungi are distinguished from almost all other fungi by the presence of 9,10-*cis*-epoxyoctadecanoic acid (epoxy C_{18}) which comprises between 1.7 and 78% of the total fatty acids in the spores studied (3). Fatty acids, as methyl esters, of *C. fusiforme* basidiospores were identified by comparison of GLC retention times with those of authentic standards and fatty acid methyl esters previously identified by GLC-MS prepared from *C. fusiforme* aeciospores (6). Nonsubstituted fatty acids are similar to those of other rust spores (4,5) and aeciospores of *C. fusiforme* (6) (Table I). Chain-lengths ranged from C_{14} to C_{18}, with C_{16} (11.7%) being the predominant saturated acid and $C_{18:2}$ (17.2%) and $C_{18:3}$ (16.1%) being the most abundant unsaturated acids. Like all uredospores, aeciospores, and teliospores of rust fungi previously reported (3,9), basidiospores of *C. fusiforme* contain high rela-

TABLE II

Major High Mass Ion Fragments in the Mass Spectra of Principal Sterols from *C. fusiforme* Basidiospores

Sterol	Molecular ion (m/e)	Base peak (m/e)	Major high mass fragments (m/e)
Stigmast-7-enol			
Free	414	414	399,379,353,314,299,271,255,246,231, 229,213
Acetate	456	313	441,439,396,394,381,379,356,341,327, 296,273,255,229,213
TMS	486	386	471,469,371,343,281,255,229,213
Stigmasta-5,7-enol			
Free	412	412	397,394,379,353,271,253
Acetate	454	394	439,379,356,353,313,253
TMS	484	484	469,396,386,381,379,343,281,253,299, 213
Ergost-7-enol			
Free	400	400	385,365,339,273,255,231,229,213
Acetate	442	442	427,382,367,315,255,229,213
TMS	472	472	457,382,367,281,255,229,213

tive proportions of the epoxy C_{18} acid (40.7%) characteristic of this group of fungi. While the fatty acid composition of aeciospores (6) and basidiospores of this species are very similar, there are certain qualitative and quantitative differences between them. C_{22}, $C_{22:1}$ and $C_{24:1}$ were tentatively identified as minor components of *C. fusiforme* aeciospores, but they were not detected in the basidiospores. Also, C_{18} and $C_{18:1}$ were present in basidiospores in approximately one-half the relative proportions found in aeciospores, whereas $C_{18:2}$ and $C_{18:3}$ were present in 1.8 to 2.7 times higher relative concentrations, respectively, than in aeciospores. Basidiospores contain about 10% less of the epoxy C_{18} acid than aeciospores.

Ergosterol is generally considered the principal fungal sterol; however, it has not been detected in rust spore extracts (3,9). Δ^7C_{29} sterols are predominant in rust fungi (9). Digitonin precipitable sterols of *C. fusiforme* basidiospores were analyzed as the free alcohol and as the acetate and TMS derivatives by GLC-MS. The principal sterol represents 56.7% of the total, and was identified as stigmast-7-enol. Molecular ions for the free alcohol and the acetate and TMS derivatives were m/e 414, 456, and 486, respectively, which suggests a monounsaturated C_{29} sterol. Ion fragments at m/e 255 in each spectra and 296 and 356 in that of the acetate derivative suggest that the single double bond is located in the ring system of the sterol (10). Two probable locations for the single double bond of the sterol ring system are the Δ^5 or Δ^7 positions. The absence of M^+-129 in the mass spectrum of the TMS derivative suggests that the double bond is not in the Δ^5 position (10,11). According to Knights (10), Δ^7 sterols seem to have no unique ions, but may be characterized by m/e 213 [M^+-(side-chain + 42 + ROH)] and m/e 229 [M^+-(side-chain + 27 + RO)], and Δ^7 sterols show a strong M^+-(side chain + 2 x H). This is true for the principal sterol of *C. fusiforme* basidiospores with m/e 271, 313, and 343 as prominent ion fragments in the spectra of the free alcohol and acetate and TMS derivatives, respectively. Also, m/e 213 and 229 are present in the spectra of this sterol.

The second most abundant sterol in *C. fusiforme* basidiospores is stigmasta-5,7-dienol, comprising 22.7% of the total. This sterol had a shorter retention than stigmast-7-enol as would be expected on a SE-30 GLC column, but was only partially resolved from it. Molecular ions from the mass spectra of the free alcohol and the acetate and TMS derivatives were 412, 454, and 484, respectively, which correspond to a C_{29} sterol with two double bonds. Both double bonds may be located in the ring system, one in the ring system and the other in the side-chain or both in the side-chain. Based on structures of known diunsaturated sterols of plant or fungal origin, the most probable structure for a sterol with two double bonds in the ring would be $\Delta^{5,7}$, or if one double bond was in the side chian it may be located in either the Δ^{22} or $\Delta^{24(28)}$ position. To our knowledge, a naturally occurring sterol with no double-bonds in the ring system and two in the side-chain has not been reported. The presence of a significant peak at m/e 253 [M^+-side-chain + ROH)] rather than m/e 255 in each spectrum suggests that both double bonds are located in the ring system. The presence of significant peak at m/e 353 in the spectrum of the free alcohol is evidence for the $\Delta^{5,7}$ structure which, according to Smith and Korn (12), is produced by the splitting of ring A [M^+-(C-1 to C-3)] and seems

to be characteristic of $\Delta^{5,7}$ sterols. This fragment is absent from the corresponding $\Delta^{7,24(28)}$ C_{29} isomer (6).

The third most abundant sterol comprising 16.0% of the total is ergost-7-enol (fungisterol). The molecular ions in mass spectra of the free alcohol and the acetate and TMS derivatives are m/e 400, 442, and 472, respectively, which are indicative of a C_{28} sterol with a single double bond in the ring structure. The absence of M^+-129 in the spectrum of the TMS derivative suggests that the double bond is not in the Δ^5 position. The presence of significant peaks at m/e 213 and 229 in each spectrum suggests that the double bond is in the Δ^7 position.

The fourth sterol of *C. fusiforme* basidiospores comprised 4% of the total and had a shorter retention time than ergost-7-enol, but was only partially resolved from it. Due to the small sample size, a definitive mass spectrum was not obtained for this sterol. However, it was tentatively identified as a C_{28} diene, with two double bonds in the ring system (m/e 253). Major high mass ion fragments of the three principal sterols of *C. fusiforme* are summarized in Table II.

Sterols of several rust species have been reported. Three of these reports concerned uredospores of *Puccinia graminis* (13-15). Weete and Laseter (15) confirmed the presence of stigmast-7-enol by mass spectrometry as the most abundant sterol of *P. graminis* uredospores, which was accompanied by a C_{29} diene and ergost-7-enol. Jackson and Frear (16) identified the Δ^7 and $\Delta^{7,24(28)}$ C_{29} sterols in flax rust (*Melampsora lini*) uredospores which were accompanied by small amounts of the $\Delta^{5,7}$ isomer. Lin et al. (17,18) identified (24Z) stigmast-7,24(28)-dienol as the principal sterol of *Uromyces phaseoli* uredospores which was accompanied by low relative amounts of the corresponding Δ^7 C_{29} isomer. At this time, it appears that rust uredospores are of two types based on sterol composition; one type contains stigmast-7-enol as the principal sterol, accompanied by ergost-7-enol, and another group has stigmasta-7,24(28)-dienol as the major sterol.

These data, combined with our previous report of *C. fusiforme* aeciospore sterols (6), represents the first comparison of the sterol composition of two different spore types of a single rust species. Aeciospores of *C. fusiforme* contains stigmasta-7,24(28)-dienol which represents ca. 98% of the total sterols, whereas the basidiospores of this species contain stigmast-7-enol, stigmasta-5,7-dienol, and ergost-7-enol, as principal sterols. The sterol composition of basidiospores is very similar to uredospores of *P. graminis* and *M. lini*. From the standpoint of host damage, basidiospores are formed on the alternate host, *Q. rubra*; aeciospores infect oak where uredia and then telia are subsequently produced. Teliospores germinate to form basidiospores, which infect primary hosts, *Pinus taeda* or *P. elliottii*. The similarity of the sterol composition between basidiospores of *C. fusiforme* and the uredospores of other rust species may be linked to the fact that uredospores and basidiospores of *C. fusiforme* are formed on the same host (oak). Both spore types are haploid, but basidiospores contain a single nucleus whereas the uredospores are dikaryotic.

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Alkyl- and Alkenylresorcinols in *Rapanea laetevirens* Seed Lipids

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ABSTRACT

The seed lipids of *Rapanea laetevirens* (Myrsinaceae) were found to contain a series of 5-alkylresorcinols. Some of these alkylresorcinols have been reported previously in other plant families, but never before in the Myrsinaceae. The various homologs were shown by mass spectrometry to have alkyl side chains that included C_{11} and C_{13} saturated as well as C_{11}, C_{13}, and C_{15} monoenoic substituents. This is the first report of the natural occurrence of tridecenylresorcinol, the homolog with the C_{13} monoenoic side chain. The positions of the double bonds in the side chains were determined by mass spectrometry of their methoxy trimethylsilyloxy derivatives. Also found were embelin and rapanone, benzoquinone pigments which occur commonly in the Myrsinaceae. The fatty acid composition of *Rapanea* seed triglycerides was not unusual, consisting mainly of 16:0 (27%), 18:1 (22%), and 18:2 (44%).

INTRODUCTION

As part of a program of investigating the composition and utility of unusual seed lipids, we have examined the seed oil of *Rapanea laetevirens* (Myrsinaceae).

The natural occurrence of 5-n-alkyl and 5-n-alkenyl resorcinols (Fig. 1) has been well documented in the literature. These compounds have been reported in the Anacardiaceae (cashew nut shell liquid) (1), Ginkgoaceae (*Ginkgo biloba*) (2), certain species of Gramineae (3), and in the Proteaceae (4,5). This paper describes the characterization of five of these alkyl and alkenyl resorcinols in the seed oil of *R. laetevirens*. This is the first report of these compounds in plants of the family Myrsinaceae, and also the first report of tridecenylresorcinol in nature.

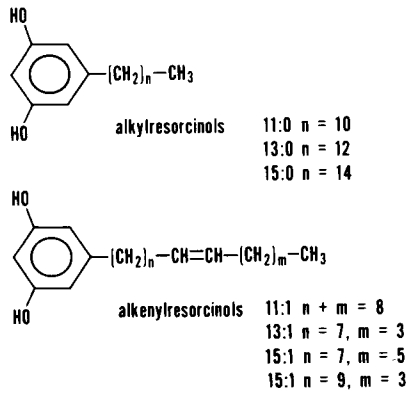


FIG. 1. Alkyl- and alkenylresorcinols from *Rapanea laetevirens* seed lipids.

EXPERIMENTAL PROCEDURES

Chromatographic Methods

Thin layer chromatography (TLC) was performed on 20 x 20 cm glass plates coated with 0.25 mm layers of Silica Gel 60F-254. Hexane-ethyl ether-acetic acid (80:20:2) was the developing solvent system. Spots were visualized by UV light or by iodine vapors.

Preparative argentation TLC was carried out on 1-mm layers of Silica Gel G impregnated with 20% $AgNO_3$ and using benzene as the developing solvent. Separated bands were located under long-wave UV light after spraying with ethanolic dichlorofluorescein. The fractions were eluted with ethyl ether. R_f values reported for each fraction are from analytical TLC which was carried out on 0.275-mm layers of the same absorbent with benzene as the developing solvent. On analytical plates, the spots were visualized by charring with sulfuric acid-dichromate solution.

Gas liquid chromatography (GLC) of the fatty acid methyl esters derived from the triglycerides was carried out on Apiezon L and LAC-2-R 446 columns as described by Mikolajczak et al. (6). GLC of other materials was carried out by one of the following methods: (a) on a Hewlett-Packard model 5750 gas chromatograph with a 1.0 m x 3.0 mm stainless steel column packed with nonpolar phase 3% OV-1 on 100-120 mesh Gas Chrom Q (Applied Science Laboratories, State College, PA), in which the temperature was programmed at 4 C/min from 150 to 400 C, or (b) on a Packard model 7401 gas chromatograph equipped with a 2.0 m x 2.0 mm glass column packed with the polar phase Silar 5-CP (Applied Science Laboratories), in which analyses were carried out isothermally at 215 C.

Spectrometric Methods

IR spectra were obtained with a Perkin-Elmer model 700 instrument as 1% $CHCl_3$ solutions in 1 mm NaCl cells. Nuclear magnetic resonance (NMR) spectra were recorded with a Varian HA-100 spectrometer on deuterochloroform solutions. Gas chromatography-mass spectrometry (GC-MS) was carried out with a DuPont 21-491-2 mass spectrometer. Samples were introduced into the mass spectrometer through a Bendix 2600 gas chromatograph and a stainless-steel jet separator. The gas chromatograph was equipped with a 2 m x 2.0 mm glass column packed with 3% OV-1 on Gas Chrom Q. The temperature was programmed 4 C/min from 190 to 250 C. The inlet was at 230 C, the transfer line and jet separator at 250 C, and the mass spectrometer source was kept at 230 C. The filament current was 250 μA and the ionizing voltage, 70 eV.

Seed Extraction

Ground *R. laetevirens* seeds were extracted 6 hr with petroleum ether (b.p. 30-60 C) in a Soxhlet extractor. Solvent was removed from the extract with a rotating evaporator.

Countercurrent Fractionation

Countercurrent distribution (CCD) (7-9) was conducted in a 200-tube Craig-Post apparatus. Seed oil (8 g) was separated by using hexane-nitroethane as the solvent system at temperatures no higher than 22 C (8,10). The tubes contained 40 ml of each phase throughout the entire distribution. After 200 transfers, upper phases were decanted into a fraction collector (one transfer per tube).

Chemical Derivatives

Methyl esters of the triglyceride fatty acids were prepared by methanolysis of the oil with HCl as catalyst (6).

Trimethylsilyl (TMS) derivatives were prepared by treatment with *bis*-(trimethylsilyl)trifluoroacetamide (BSTFA) (Regis Chemical Co., Morton Grove, IL). The products were subjected directly to GLC and GC-MS without purification.

Acetates of phenols were prepared by reacting the samples with acetic anhydride in pyridine (11).

Methoxy trimethylsilyloxy derivatives were prepared by a three-step process. The olefinic starting materials were epoxidized with *m*-chloroperbenzoic acid (12). The methoxyhydroxy derivatives were formed by the action of BF_3-MeOH upon the epoxides (13,14). Treatment of these derivatives with BSTFA yielded the methoxy trimethylsilyloxy derivatives (15).

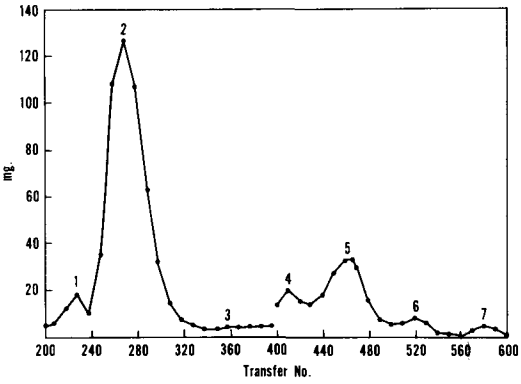
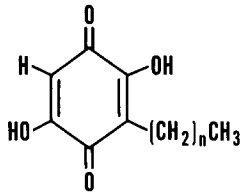


FIG. 2. Countercurrent distribution of *Rapanea laetevirens* seed oil. Solvent system, hexane-nitroethane; 40 ml upper phase decanted, transfers 200-399; fundamental series, transfers 400-600. Weight peaks are identified by number as discussed in text.



Embelin, n = 10
Rapanone, n = 11

FIG. 3. Benzoquinones from *Rapanea laetevirens* seed lipids.

RESULTS AND DISCUSSION

Preliminary Examination

The petroleum ether extract was a deep red semisolid which represented 21% of the weight of the ground seed. IR absorbance was at 1370, 1640, and 3360 cm^{-1} (-OH) in addition to the usual peaks for triglycerides. GLC of the methyl esters prepared from the oil showed the fatty acid content to be 16:0, 27.2%; 18:0, 2.4%; 18:1, 21.5%; 18:2, 43.5%; 18:3, 1.3%.

Countercurrent Fractionation

The weight distribution achieved by CCD of 8 g of *R. laetevirens* seed lipids is indicated in Figure 2. Integration of the weight curve showed the following distribution (fraction no., wt %): 1, 5.2%; 2, 63.3%; 3, 2.6%; 4, 5.9%; 5, 17.7%; 6, 3.7%; 7, 1.6%. TLC and IR of the combined products from fraction 2 indicated that it contained mainly triglycerides. The IR

spectra of fractions 5 and 6 showed strong absorbance at 3360 cm^{-1} (OH). Fractions 5 and 6 were chosen for further investigation because of their apparent chemical novelty. The other fractions were not investigated further.

CCD Fraction 5: Embelin and Rapanone

The material was recovered as orange-colored plates, m.p. 139-141 C (uncorrected). It was compared to embelin (m.p. 141-143 C) (Fig. 3) from *Myrsine africana* and found to have similar properties in IR, UV, and TLC. MS (probe) showed the presence of small amounts of rapanone (ca. 15%) mixed with the embelin.

CCD Fraction 6: Alkyl- and Alkenylresorcinols

Fraction 6 showed significant IR absorbance maxima at 3360 cm^{-1} (-OH). A probe MS showed the characteristic spectrum of alkyl- or alkenylresorcinols (16). The base peak was at m/e 124 $[(HO)_2C_6H_3CH_3^+]$ along with three apparent molecular ions (M^+) at m/e 264, 292, and 318. These M^+ values indicated that the side chains were undecyl, tridecyl, and pentadecenyl. (Hereafter the various alkyl and alkenyl resorcinols will be designated by length and degree of unsaturation of their alkyl or alkenyl side chain, i.e., C11:0, C13:0, and C15:1, respectively).

These observations were confirmed by preparation and analysis of the TMS and diacetate derivatives. GC-MS of the TMS ethers showed significant ions that would be expected for the TMS derivatives of the alkyl- and alkenylresorcinols. The spectrum for each of the three major components had a base peak at m/e 268 $[(TMSO)_2C_6H_3CH_3^+]$ along with the expected M^+ at 408, 436, and 462.

The IR spectrum of the acetates showed a new peak at 1380 cm^{-1} along with the disappearance of the hydroxyl absorbance at 3360 cm^{-1}. GLC gave peaks whose mass spectra indicated acetate derivatives of the following alkyl- and alkenylresorcinols (GLC area percent in parentheses): 11:0 (15.1), 11:1 (tr), 13:0 (22.3), 13:1 (4.5), 15:0 (6.5), 15:1 (44.9). The remaining components (6.7%) were not identified. The mass spectra did not give noticeable molecular ions in each case, but always contained prominent M-42 and M-84 ions. These ions corresponded to loss of one or two molecules of ketene during cleavage of acetate groups through a McLafferty-type rearrangement (17). The base peak at m/e 124 and an ion of lower intensity at m/e 166 can also be attributed to the same type cleavage from the ion $[CH_3COO]_2C_6H_3CH_3^+$, m/e 208. Analogs of the latter ion predominate in MS of TMSO or methoxy resorcinols but appeared here only as minor fragments.

The position of unsaturation could not be determined directly by MS of the resorcinols with the side chain underivatized. However, preparation of the methoxy trimethylsilyloxy derivatives of the olefins afforded a convenient method for double bond location by MS. Before derivatizing the double bonds, separation of acetylated resorcinols with unsaturated side chains from other resorcinols was accomplished by argentation TLC and gave three fractions containing resorcinols [(Fraction) R_f]: (1) 0.15, (2) 0.33, (3) 0.55. These fractions migrated between bands containing diglycerides (lower R_f) and unknown materials (higher R_f) in amounts depending on CCD tube examined. IR spectra showed that these fractions had significant absorbance at 1380 cm^{-1}. GLC and GC-MS showed that fraction 1 contained about 20% of the alkenyl resorcinols C11:1, C13:1, and C15:1 with the remainder being mainly acetates of diglycerides. Fraction 2 was exclusively the 13:1 and 15:1 homologs, while fraction 3 consisted of the saturated alkyl resorcinols 11:0, 13:0, and 15:0.

Epoxidation of fraction 2 followed by methanolysis and silylation provided methoxy trimethylsilyloxy derivatives without cleavage of the alkenyl side chain (15). Both positional isomers were formed at the site of the original double bond and the acetoxy groups on the aromatic ring were replaced by TMSO groups in the process. GLC (OV-1 column) gave peaks whose mass spectra are summarized in Table I.

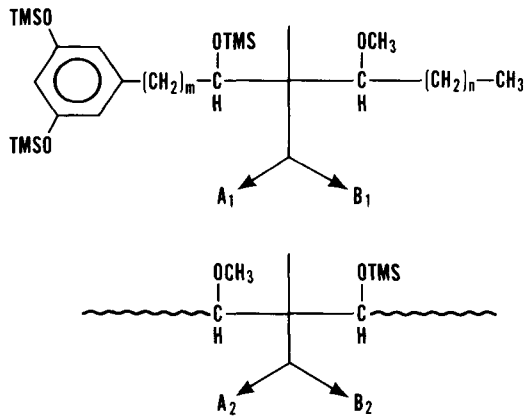
In the case of the derivative obtained from the C13:1 monoene, the ions at m/e 395 and 453 locate the double bond at $\Delta 8$ (counting from the aromatic ring). The ions at m/e 101 and 159 fix the position at $\omega 5$. The molecular ion (m/e 554) was in accord with these observations. The derivative of the 15:1 resorcinol gave a peak with a shoulder. MS at the apex and at the shoulder revealed two isomers, one $\omega 5$ and the other $\omega 7$. In addition, all of these spectra gave ions at m/e 73 (TMS) and 268 $[(TMSO)_2C_6H_3CH_3^+]$. The latter two are characteristic of silylated resorcinols.

The IR spectrum of the original CCD fraction 6 did not show the absorbance at 970 cm^{-1} characteristic of olfeinic *trans* double bonds. Accordingly, the double bonds must have the *cis* configuration.

The position of the two hydroxyl groups on the aromatic ring was shown by MS and NMR to be 1,3- (resorcinols) rather than 1,2- (catechols) or 1,4- (hydroquinones). Alkylresorcinols have been shown by other investigators to give a base peak representing the ion m/e 124 (16,18), whereas the alkylcatechols

TABLE I

Ions Observed on Gas Chromatography-Mass Spectrometry of Methoxy Trimethylsilyloxy Derivatives of Alkenylresorcinols



Underivatized monoene[a]	M[b]	Double bond position (Δ)	m	A_1[c]	A_2[c]	Double bond position (ω)	n	B_1[c]	B_2[c]
C13:1	554 (11)	8	7	453 (60)	395 (25)	5	3	101 (13)	159 (100)
C15:1[d]	582 (10)	8	7	453 (59)	395 (16)	7	5	129 (12)	187 (72)
C15:1[e]	582 (3)	10	9	481 (19)	423 (14)	5	3	101 (12)	159 (100)

[a]Carbon side chain length:degree of unsaturation.

[b]Molecular ion (percent base peak in parentheses).

[c]Mass spectral fragments (percent base peak in parentheses).

[d]Mass spectrum taken at apex of gas liquid chromatographic peak.

[e]MS taken at should of GLC peak.

(urushiols) give a base peak representing the ion $(HO)_2C_6H_3CH_2^+$ m/e 123 (19). Similarly, the base peak for the TMS derivatives was at m/e 268, but the TMS derivatives of the alkylcatechols give a base peak at m/e 267 $[(TMSO)_2C_6H_3CH_2^+]$ (20). The ions at m/e 123 and 267 result from fission β to the benzene ring while those at m/e 124 and 268 seemingly are caused by β-cleavage with a rearrangement involving transfer of one hydrogen atom (16,19).

In the NMR spectrum of the acetates, the aromatic protons appear as two doublets (J=2Hz) at δ6.70 (1H) and 6.77 (2H). This coupling constant indicates that all three aromatic protons must be meta to each other (21), so that the aromatic substituents must have a 1,3,5-relationship as in the 5-alkyl (and alkenyl) resorcinols (22,23).

This is the first report of a natural C13:1 alkenyl resorcinol.

ACKNOWLEDGMENT

Methyl esters were prepared and analyzed by J.W. Hagemann; NMR by L. Tjarks.

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SHORT COMMUNICATION

Identification of the *trans* Isomers of Octadecenoic Acid in Human Milk

ABSTRACT

The presence of *trans* octadecenoic acid isomers was detected and analyzed in 24 hr pooled samples of human milk. Amounts of the *trans* isomers of the C_{18} monoenes ranged from 2% to 4% of the total fatty acids. For purposes of comparison, three commonly used brands of infant formula were also analyzed and found to contain 0.1% to 1.3% of *trans* monoene isomers. Data indicate that breast-fed and bottle-fed infants are receiving minimal levels of *trans* fatty acids via milk.

INTRODUCTION

The influence of dietary fat on the fatty acid composition of human milk was first demonstrated by Insull and associates (1,2). They were able to elevate milk polyenoic acid content by substituting corn oil for lard in the maternal diet. Risek et al. (3) reported that between 1957 and 1972 there were characteristic changes in American fat consumption representing a marked shift from animal to vegetable sources. Human milk fatty acid patterns have been observed to reflect these changes in fat consumption (4). A comparison of recent analyses with those reported by Insull and associates in 1959 (1,2) shows a lower proportion of 12:0, 14:0 acids, similar proportions of 16:0, slightly more 16:1, 18:0, 18:1 and almost twice as much 18:2.

Many commercially available sources of vegetable fat are prepared by the process of partial hydrogenation, and this practice results in the formation of *trans* double bonds in monounsaturated and diunsaturated fatty acids. Thus, associated with the shift from animal to vegetable sources of fat is an increased consumption of *trans* fatty acids, estimated to be 8% of visible fat intake (5). The present study was undertaken to determine whether the most prevalent isomerization products of partially hydrogenated fats, *trans* monoenes, were present in the milk of lactating women.

METHODS

In July, 1976, three Champaign, IL women who had successfully established lactation each provided a 24 hr pooled milk sample. Subjects A and B were in their 3rd month of lactation, while Subject C was in her 15th month. All samples were collected by manual expression, frozen after collection, and transported to the laboratory in the frozen state for analyses which were performed immediately. For purposes of comparison, three commonly used brands of infant formula also were purchased for analyses.

The total lipids of human milk and formula were extracted by the Folch method (6) using chloroform-methanol (2:1 v/v). The lipid fraction was dried under vacuum and the corresponding methyl esters were prepared by refluxing the total lipid with anhydrous methanol containing 3% H_2SO_4 (v/v). Methyl esters were isolated by extraction with purified hexane, and dried over anhydrous sodium sulfate. The esters so prepared were separated by gas chromatography on a 20 ft. x 1/8 in. S.S. column packed with 15% OV-275 on 100/120 mesh Chromosorb P AW-DMCS (Supelco Inc., Bellefonte, PA) (7,8). The column conditions were as follows: Temp. 220 C, Injecter 250 C, Detector 350 C, Flow 8 ml/min/N_2. Sample size was 1 μl injected containing ca. 10 μg ester. Quantitation was achieved by the electronic integrator of the HP5830 instrument employed (Hewlett Packard, Sacramento, CA). This was checked and calibrated with appropriate standards.

RESULTS AND DISCUSSION

The results of the human milk analyses are presented in Table I. *trans* Monoenes were detected in amounts ranging from 2% to 4% of the total fatty acids. These data indicate that associated with the current trends of high vegetable fat consumption is not only a high polyenoic acid content of human milk but also detectable levels of the *trans* isomers of oleic acid. Far fewer *trans* isomers were found in the three brands of infant formula analyzed (Table II). Only 0.1% to 1.2% of the total fatty acids in formula are the 18:1 *trans* isomers. The fat content of these products is normally a mixture of vegetable oils, such as corn, soy,

TABLE I

Fatty Acid Composition of Human Milk Lipids

Fatty acid	Subject A	B (Relative %)	C
8:0	0.5	0.7	.4
10:0	2.0	0.7	1.4
12:0	5.4	4.7	6.3
14:0	5.9	6.8	7.3
16:0	19.3	21.2	18.6
16:1	5.4	3.2	2.8
18:0	6.4	7.1	7.2
18:1 *trans*	2.1	4.0	3.2
18:1 *cis*	34.1	30.1	31.0
18:2	13.2	13.9	17.1
18:3	1.2	1.0	0.8
20:4	0.3	0.2	0.2
Others[a]	4.2	6.4	3.2

[a]Odd Chain and Minor Components.

TABLE II

Fatty Acid Composition of Infant Formula

Fatty acid	Product[a] SMA	EN (Relative %)	SIM
10:0	2.0	1.1	4.9
12:0	14.1	9.1	33.6
14:0	6.9	3.7	11.6
16:0	13.9	10.9	9.6
16:1	1.9	0.2	0.2
18:0	7.8	4.2	3.5
18:1 *trans*	1.3	0.2	0.1
18:1 *cis*	36.2	19.5	12.1
18:2	14.3	44.6	21.4
18:3	0.8	4.9	2.0
Other[b]	0.8	0.9	0.3

[a]SMA = SMA (Wyeth Laboratories, Radnor, PA), EN = Enfamil (Mead Johnson Laboratories, Evansville, IN), SIM = Similac (Ross Laboratories, Columbus, OH).

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safflower, and coconut oils. The low level of elaidic acid found suggests that they were only lightly hydrogenated.

The significance of *trans* fatty acids in the American diet is unknown. Vergroesen (9) reported the 35% elaidic acid in formula which provided 40% of dietary energy as fat increased serum cholesterol levels but with the exclusion of dietary cholesterol, no effect was observed. However, Mattson et al. (10) fed comparable levels of elaidic acid with 500 mg cholesterol/day and found no elevation in plasma cholesterol. It is unlikely that the low levels of elaidic acid in human milk and infant formula would be associated with altered blood lipid patterns.

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Prevention of Obesity in A^{vy}/a Mice by Dehydroepiandrosterone

TERENCE T. YEN, JEAN A. ALLAN, DONOVAN V. PEARSON, JUNE M. ACTON, and **MARK M. GREENBERG,** The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46206

ABSTRACT

Dehydroepiandrosterone, a mammalian glucose-6-phosphate dehydrogenase inhibitor, prevented A^{vy}/a mice from becoming obese. Decreased accumulation of triacylglycerol accounted for a large portion of the weight difference between treated and control A^{vy}/a mice. Hepatic lipogenesis as measured by ^3H_2O incorporation into total lipid was less in the dehydroepiandrosterone-treated mice. Dehydroepiandrosterone did not suppress appetite and had no apparent toxic effects at the doses used, and its weight controlling effects were reversible upon withdrawal of treatment.

INTRODUCTION

Dehydroepiandrosterone (DHA), a steroid synthesized and secreted by the adrenal (1,2), is an inhibitor of mammalian glucose-6-phosphate dehydrogenase (G6PDH) (3-12), one of the enzymes supplying the NADPH required for fatty acid synthesis (13). Obese individuals excrete less DHA than the nonobese (14,15), and the urinary output of DHA increases during weight loss (15). These observations have led to the hypothesis that DHA may play a regulatory role in obesity (16). One possibility is that a higher concentration of DHA would decrease lipogenesis by lowering the availability of NADPH through the inhibition of G6PDH. However, there is no direct evidence to support this hypothesis.

Inhibition of erythrocyte and liver G6PDH by DHA has been demonstrated in vivo with humans and rats (17-20). In adrenal homogenates, apparent inhibition of G6PDH by DHA lowered the concentration of NADPH and thereby decreased glucose metabolism (21), an effect that could be reversed by addition of yeast G6PDH which is not inhibited by DHA (21). Furthermore, Ziboh et al. (22) reported that DHA inhibited synthesis of lipids from acetate by rat skin in vitro.

In spite of the work cited above, no direct evidence is available to suggest that DHA may be involved in the regulation of weight and the prevention of obesity. The data presented in this report show that DHA, possibly through an inhibition of hepatic lipogenesis, prevents viable yellow obese mice from becoming obese. A preliminary report of this investigation was published elsewhere (23).

MATERIALS AND METHODS

Two genotypes of mice of the *agouti locus* from our inbreeding colony were used in this study. They were the viable yellow obese mice (A^{vy}/a), genetically destined to become obese, and the black, recessive normal homozygotes

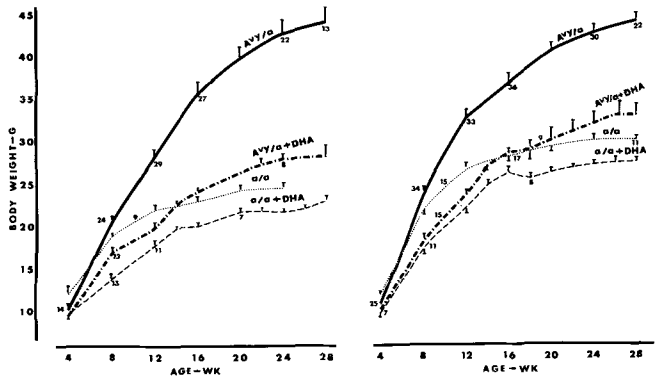

FIG. 1. Body weight of female (left panel) and male (right panel) A^{vy}/a and a/a mice given DHA, 500 mg/kg, p.o., in sesame oil three times weekly. The A^{vy}/a mice serving as controls were contemporary untreated mice combined with mice treated with sesame oil. The a/a mice serving as controls consisted of contemporary untreated mice only. All treatments started at the time of weaning and lasted the entire period shown in the graph. The curves illustrate mean body weight ± S.E. The numbers on the curves indicate tne number of mice for the point. If no number is shown, the number of mice is the same as that for the previous point.

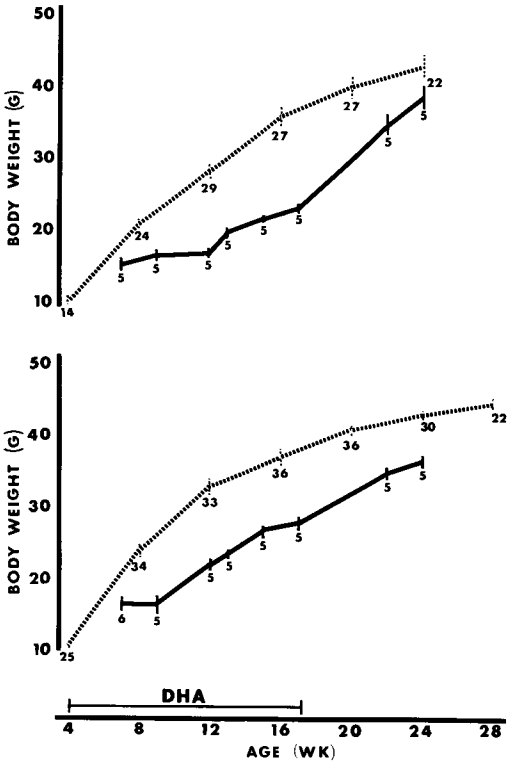

FIG. 2. Body weight of female A^{vy}/a mice (top panel) and male A^{vy}/a mice (bottom panel) given DHA, 500 mg/kg, p.o., in sesame oil three times weekly from 4 to 17 wk of age (solid line). At 17 wk of age, the treatment was discontinued. The A^{vy}/a mice serving as controls were contemporary untreated mice combined with mice treated with sesame oil (dotted line). The curves illustrate mean body weight ± S.E. The numbers on the curves indicate the number of mice for the point.

TABLE I

Food Consumption of DHA- and Vehicle-Treated A^{vy}/a Mice

Treatment	No. of mice	Food consumption g/mouse/day
Control	8♂ & 4♀	3.8 ± 0.1 (42)[a]
Control	5♂	3.5 ± 0.2 (25)
500 mg/kg, p.o.	7♂	4.7 ± 0.3 (12)
0.2% feed	5♂ & 5♀	3.6 ± 0.2 (57)

[a]Mean ± S.E. (no. of measurements).

(*a*/*a*) of the VY strain. Mice were fed Purina Laboratory Chow and had access to water ad libitum. Mice sacrificed for analysis had feed and water available until the time tritiated water was injected. The animal room was lighted from 6 am to 6 pm.

Procedures for analyzing triacylglycerol content and for measuring in vivo lipogenesis rates with tritiated water have been defined elsewhere (24). Chemicals and standards for all assays were from the same suppliers as described before (24). DHA was purchased from Searle Chemicals Inc., Chicago, IL. Emulphor EL-620 (General Aniline and Film, New York, NY), a polyoxyethylated vegetable oil, was used to facilitate the suspension of DHA in saline for parenteral injections.

Student's t-test was used in all statistical analyses of the data.

RESULTS

DHA given at 500 mg/kg, p.o., in sesame oil three times weekly controlled weight gain of both female and male A^{vy}/a mice (Fig. 1). The weights of the treated A^{vy}/a mice were only slightly greater than that of the control, lean *a*/*a* mice. Treatment of *a*/*a* mice with 500 mg/kg, p.o., DHA also reduced the weight gain of both female and male *a*/*a* mice (Figure 1), but the effect was not so pronounced as with A^{vy}/a mice.

The effect of DHA treatment was reversible. After the treatment was withdrawn, the weight of the mice increased and eventually approached that of mice not treated with DHA (Fig. 2). This was more apparent with the female mice than with the male.

DHA was also effective with both male and female A^{vy}/a mice when given in a diet containing 0.2% of the compound but was effective only with female A^{vy}/a mice when given at 150 mg/kg, p.o., thrice weekly or 10 mg/kg, i.p., thrice weekly. About 75 A^{vy}/a mice genetically destined to be obese were prevented from becoming obese by these treatments. The fact that parenteral injections of DHA are more effective than dietary DHA suggests that the compound is poorly absorbed.

The possibility of appetite suppression by DHA was investigated by monitoring the food consumption of the treated mice either periodically or throughout the entire treatment period (Table I). The food consumption of the DHA-treated A^{vy}/a mice was within or slightly above the normal range. Indeed, since the DHA-treated A^{vy}/a mice were lighter in weight, their food consumption on body weight basis was actually greater than that of vehicle-treated A^{vy}/a mice. A single injection of DHA at a dose as high as 32 mg/kg, i.p., also had no effect in an appetite-suppression test. The mice did not eat well when fed a diet containing 0.5% DHA. At that concentration in the diet, the dose would be equivalent to about 800 mg/kg of DHA per day. Kandutsch et al. (25) found androstanolone to suppress appetite at this dose.

TABLE II

Triacylglycerol (TG) Content and Incorporation of ^3H_2O into Total Lipid[a] in Liver and Carcass[b] of A^{vy}/a and a/a Mice

Treatment	N	Body weight (g)	μmoles TG / g body weight	Liver weight (g)	μmoles TG / g liver	μmoles ^3H_2O incorporated[a] into total lipid in carcass per g body weight	μmoles ^3H_2O incorporated[a] into total lipid in liver per g liver weight
			Male A^{vy}/a mice, 8 to 9 mo old				
Sesame oil, p.o.	7	49.6 ± 0.7[c]	358 ± 38	2.50 ± 0.06	75 ± 4	3.5 ± 0.1	57 ± 4
DHA, 500 mg/kg, p.o.	8	36.6 ± 1.5	179 ± 12	1.69 ± 0.07	20 ± 2	3.5 ± 0.2	33 ± 1
			Male a/a mice, 5 to 8 mo old				
None	8	29.9 ± 0.9	72 ± 8	1.23 ± 0.04	10 ± 1	2.6 ± 0.1	31 ± 4
			Female A^{vy}/a mice, 8½ to 9 mo old				
Sesame oil, p.o.	4	51.1 ± 3.3	442 ± 37	2.15 ± 0.21	61 ± 9	4.5 ± 0.6	61 ± 3
DHA, 500 mg/kg, p.o.	5	30.7 ± 1.2	202 ± 48	1.42 ± 0.06	19 ± 4	3.7 ± 0.4	42 ± 7
			Female a/a mice, 5 mo old				
None	4	23.1 ± 0.3	91 ± 6	1.03 ± 0.05	13 ± 3	2.7 ± 0.1	24 ± 1

[a]Lipogenesis rates in vivo were determined by measuring the incorporation of ^3H_2O into total lipid 1 hr after a trace amount of ^3H_2O was injected into the tail vein of the mouse. The rate of incorporation was calculated on the basis of dilution of ^3H_2O by total body water space which was estimated by the plasma radioactivity of each mouse, assuming water constitutes 90% of plasma (26).

[b]Carcass = entire body minus liver and most of the blood.

[c]All data are expressed as mean ± S.E.

The action of DHA on obesity would not appear to be due to a general toxic effect. The acute LD_{50} of DHA in mice is between 1 and 2 g/kg, s.c. Twenty A^{vy}/a and a/a mice on chronic DHA treatment were autopsied. No pathological changes were found that would contribute to the observed weight control.

For both male and female A^{vy}/a mice treated with DHA, carcass triacylglycerol contents were greatly reduced from that of the sesame oil-treated A^{vy}/a mice of the same sex (male: $p<0.0005$; female: $p<0.005$) (Table II).

DHA treatment reduced the liver weight (male: $p<0.0005$; female: $p<0.01$) but did not significantly change the liver weight/body weight ratio (Table II). DHA did, however, lower the hepatic triacylglycerol content of both male ($p<0.0005$) and female ($p<0.005$) A^{vy}/a mice (Table II).

From the data on the liver and carcass triacylglycerol content, it is possible to calculate the suppression of triacylglycerol deposition as a percentage of body weight change due to DHA. For male A^{vy}/a mice, 71% of the weight difference could be attributed to the difference in the amount of triacylglycerol deposited; for female A^{vy}/a mice, 65%. Since the female A^{vy}/a mice had higher triacylglycerol content than male A^{vy}/a mice of the same age (Table II), the inhibition of deposition due to DHA in terms of absolute amount of triacylglycerol was substantially greater in female A^{vy}/a mice than in male A^{vy}/a mice.

The lipogenesis rates of both the carcass and liver of A^{vy}/a mice were much higher than those of a/a mice (Table II). In both male and female A^{vy}/a mice, DHA significantly decreased the hepatic lipogenesis rates (male: $p<0.0005$; female: $p<0.05$) but did not affect the carcass lipogenesis rates (Table II).

DISCUSSION

A decrease of triacylglycerol content, both in the liver and in the carcass, accounts for most of the weight difference between treated and control mice. This decrease could have been caused by a lower rate of hepatic lipogenesis in the treated mice. Treatment with DHA was not found to be very effective in *ob/ob* mice, *db/db* mice and adult A^{vy}/a mice that were already obese (results not presented). We have demonstrated previously that *ob/ob* and *db/db* mice have higher lipogenesis rates even when they are young (24). In contrast, A^{vy}/a mice do not have higher hepatic lipogenesis rates until they mature. Perhaps treating A^{vy}/a mice from weaning allows sufficient time to prevent lipogenesis from becoming abnormally high with maturity.

At lower doses, DHA was more effective on female A^{vy}/a mice than on male A^{vy}/a mice. Since female A^{vy}/a mice are more obese than male A^{vy}/a mice (as shown by the triacylglycerol content), a reduction of the same percentage of triacylglycerol content would affect the weight of a female A^{vy}/a mouse more than that of a male A^{vy}/a mouse.

The control of obesity of A^{vy}/a mice is another piece of evidence suggesting that DHA may play a physiological role in regulating weight. Pharmacologically, we believe that this is the first demonstration of control of obesity with a chemical agent that probably acts on some step of metabolism (G6PDH?) instead of on appetite. Further studies are necessary to determine the mechanism responsible for these effects of DHA which may point the way to the metabolic control of human obesity.

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Fatty Acid Compositions of Lipid Fractions from Vegetative Cells and Mature Sorocarps of the Cellular Slime Mold *Dictyostelium discoideum*

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ABSTRACT

A wild-type strain of *Dictyostelium discoideum* was grown upon *Aerobacter aerogenes*. Fatty acid compositions of lipid fractions and of total lipids obtained from vegetative amoebae and mature sorocarps were compared. Fatty acids isolated from vegetative cells were found to include large quantities of 17- and 19-carbon cyclopropane fatty acids while straight-chain, saturated fatty acids represented only 10% (w/w) of total fatty acids. These cyclopropane fatty acids appear to be derived from ingested bacteria and are preferentially incorporated into neutral lipids of the slime mold. Development of amoebae to mature sorocarps is accompanied by a substantial decrease in cyclopropane fatty acid content and a concomitant increase in unsaturated fatty acids, mostly as octadeca-5,11-dienoic acid. The Δ-22 stigmastenyl ester fraction is the richest source of this acid. Fully 65% of the fatty acids in this fraction are the octadecadienoate.

INTRODUCTION

The cellular slime mold *Dictyostelium discoideum* derives most if not all of its subsistence in the wild from bacteria. Starvation triggers a well characterized pattern of differentiation which begins with the aggregation of a homogeneous population of individual amoebae and culminates with the formation of a multicelled sorocarp consisting of viable spore cells and vacuolated stalk cells (1,2). Both fatty acid content (3,4) and biosynthesis (5) in an aggregateless mutant of *D. discoideum* have been described by Davidoff and Korn, who identified two new fatty acids, *cis,cis*-5,11-octadecadienoic and *cis,cis*-5,9-hexadecadienoic acids (3). Neutral lipid components of a wild-type strain (NC-4) are known to undergo major quantitative changes during development, the most striking of which is the accumulation of Δ22-stigmastenol fatty acyl esters (6). Shifts in phospholipid composition have also been observed, particularly during aggregation, at the onset of cell-cell adhesion, and preculmination, when spore structure appears (7). Despite such information about fatty acid composition at the amoebic stage and the changes in the bulk fatty acyl esters during differentiation, little has been reported concerning the distribution and shifts of fatty acids in the ester fractions over the course of development. This communication compares the fatty acids contained in the major lipid constituents of amoebae and mature sorocarps and demonstrates that, while amoebae tend to resemble the ingested bacteria in fatty acid content, sorocarps reflect the peculiarities of *D. discoideum* fatty acid metabolism.

EXPERIMENTAL PROCEDURES

A haploid wild-type strain of *D. discoideum* (NC-4) was cultured on agar plates with *Aerobacter aerogenes* and left to develop to the desired stage before harvesting, as described previously (6). Bacteria were grown and harvested under identical conditions, without slime mold, to provide a source of bacterial lipid produced in a comparable environment (8). Total lipids were extracted by a modification of the method of Folch et al. (9); nonpolar and polar lipids were separated by elution from silica gel columns with chloroform and methanol (10); and the nonpolar (neutral) lipid fraction was resolved into sterol, glyceride, fatty acid, and sterol ester components as described earlier (6). Preparative separation of sterol esters was accomplished by elution from silicic acid with a benzene-hexane mixture (11) with monitoring by argentation thin layer chromatography (TLC) (12). Esters were saponified in alcoholic KOH and the extracted fatty acids were esterified with diazomethane. The methyl esters of unsaturated fatty acids were separated from those of saturated fatty acids by argentation chromatography (13), and the latter were fractionated further into branched and unbranched chain esters by chromatography on urea cyrstals, using iso-

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octane-methanol elution (14). A mixture of two saturated branched chain fatty acid methyl esters free of other contaminants, as evidenced by gas liquid chromatography (GLC) in an EGSS-X column, was obtained; the two were separated by countercurrent distribution, using an H.O. Post 200-tube apparatus with acetonitrile as the stationary phase and hexane as the mobile phase (15).

Unsaturated fatty acid methyl esters were hydrogenated by the procedure of Siggia (16). Positions of double bonds were determined by periodate-permanganate cleavage (17) followed by methylation of the resultant dicarboxylic acids and identification of the esters by GLC. Fatty acid methyl esters from the various fractions described above were identified by GLC, using a Varian Aerograph gas liquid chromatograph and either a 6 ft x ¼ in. EGSS-X column (Varian Aerograph, 10% EGSS-X on 100/120 mesh Gas-Chrom P) or a 6 ft x ¼ in. SE-30 column (3% SE-30 on 100/120 mesh Varaport 30). A linear temperature gradient of 4 C/min from 120 C to 200 C was used with the former and a gradient of 6 C/min from 150 C to 300 C with the latter; nitrogen was the carrier gas. Mass and nuclear magnetic resonance (NMR) spectra were obtained with an AFI Scientific Apparatus, Inc. MS 9 mass spectrometer and a Varian HA 100 IL NMR spectrometer.

RESULTS AND DISCUSSION

In the course of identification of fatty acids from lipids of vegetative amoebae by GLC, two components with anomolous retention times were noted on both EGSS-X and SE-30 columns. Both were unaffected by hydrogenation or periodate-permanganate oxidation; on fractionation, as described above, both were isolated in the saturated, branched chain fraction. Since both were also found in large quantities in *A. aerogenes* lipids, they were suspected of being cyclopropane derivatives of palmitic and stearic acids (18). Mass spectra of pure samples obtained by countercurrent distribution gave parent peaks at m/e = 310 and 286; NMR spectra contained a multiplet at 9.4τ, characteristic of the *cis* cyclopropane moiety (19). The position of the cyclopropane ring was not located, but the two acids are probably *cis*-9,10-methylene hexadecanoic and *cis*-11,12-methylene octadecanoic acids, as found in *Escherichia coli* (18).

Sterol esters from 3-day sorocarps were resolved into three fractions by silicic acid chromatography, one containing saturated fatty acid esters, a second monounsaturated esters, and a third diunsaturated esters. Periodate-permanganate oxidation of isolated monounsaturated fatty acids produced azelaic and undecanedioc acids in quantities proportional to the corresponding C_{16} and C_{18} acids; a quantity of heptanoic acid comparable to the total fatty acid present was found, but no octanoic or nonanoic acid was detected, indicating that the monounsaturated fatty acids were the hexadeca-9-enoic and octadeca-11-enoic acids. Oxidation of dienoic acid methyl esters yielded glutaric and adipic acids in addition to heptanoic, but succinic was not detected, probably because of the difficulty of extracting succinic acid from the aqueous oxidation mixture (4). The amount of adipic corresponded to the C_{18} dienoic acid while glutaric corresponded to the total C_{16} plus C_{18} dienoic acids. These results are consistent with the assumption that the major diunsaturated fatty acids are the hexadeca-5,9-dienoic and octadeca-5,11-dienoic acids, as reported by Davidoff and Korn (3,4). Small amounts of methyl nonanoate were also detected, suggesting the presence of some octadeca-5,9-dienoic acid, but at a level less than 5% of the 5,11-dienoic acid. The identity of the sterol as stigmast-22-en-3β-ol was verified by comparison of retention times on a SE-30 GLC column and mass spectrographs, using the unesterified stigmastenol isolated and purified from *D. discoideum* as a reference (21-23). The methyl ester of the fatty acid released on saponification of a pure C_{18}-dienoic fatty acyl fraction of sterol ester was comparable to but significantly different from methyl linoleate in retention time on an EGSS-X GLC column and in mass spectrographic patterns (24). The combined evidence indicates that the predominant species in the sorocarp sterol ester fraction is stigmast-22-en-3β-yl octadeca-5,11-dienoate. The rapid increase in sterol ester production during culmination and the finding that newly synthesized stigmastenol is preferentially incorporated into this fraction (6) suggest that newly synthesized unsaturated fatty acids may be preferentially incorporated into sterol esters as well, although the possibility that the fatty acids are rapidly desaturated after esterification cannot be ruled out.

All neutral lipid components shown to contain fatty acid esters and total polar lipids isolated from 3-day sorocarps as well as triglycerides and total lipid isolated from vegetative amoebae were saponified; the resultant fatty acids were extracted into ether under acidic conditions, methylated, and identified and quantitated by GLC before and after argentation TLC and periodate-permanganate oxidation. Quantitative comparisons of the

TABLE I

Fatty Acid Composition of *Aerobacter aerogenes*, Vegetative Amoebae, and 3-Day Sorocarps[a]

			Amoebae										
	A. aerogenes		Literature[b]					Sorocarp					
Fatty acid	Lit.[b]	Total	NL[c]	PL	Total	TG	Total	FFA	TG	SE	NL	PL	Total
14:0	10	18				5	1	tr	tr			tr	
15:0	1	2				1	3	tr	1		tr	4	3
16:0	38	27	24	9	15	8	5	11	4	2	5	8	7
16:1	4	8	4	8	6	8	9	8	4	4	5	7	7
16:2			5	7	6	8	10	7	5	6	6	6	6
16cyc	25	26				37	20	3	6	6	5	5	5
17:0			3	3	3	tr		tr	1	1	1	3	2
18:0			31	2	14	2	1	4	1	4	3	tr	1
18:1	9	5	17	27	23	10	20	21	22	10	15	33	29
18:2			14	37	28	16	24	32	46	65	53	30	35
18cyc	6	3				5	7						
19:0						tr							
20:0	tr	8				tr		2		tr			tr
Total	99	99	98	93	95	100	100	88	90	98	93	96	95
% sat.	55	57	58	14	32	16	10	17	7	7	9	15	13
% 1 unsat.	44[d]	42	21	35	29	60	56	32	32	20	25	45	41
% 2 unsat.	0	0	19	44	34	24	34	39	51	71	59	36	41

[a]Relative areas of recorder peaks were expressed as percent of total area.

[b]Literature values for amoebae neutral and polar lipids are from Davidoff and Korn (4) and *Aerobacter aerogenes* total fatty acids from O'Leary (18).

[c]Abbreviations are: PL, phospholipids; FFA, free fatty acids; TG, triglycerides; SE, sterol esters; NL, neutral lipids; and tr, trace amounts.

[d]Cyclopropane fatty acids were tabulated with monounsaturated fatty acids due to their unsaturated configurations.

GLC patterns provided a means of calculating the distribution of saturated, monounsaturated, diunsaturated, and cyclopropane fatty acids of different chain lengths. The results are summarized in Table I. The predominant fatty acid of both vegetative cells and sorocarps is the octadecadienoic acid. As shown above, at least 95% of this dienoic acid is the 5,11 isomer in the sterol ester of the sorocarp. The behavior of the methyl C_{18} dienoate from other ester fractions is identical to that derived from the sterol ester in GLC and is clearly distinguishable from the behavior of methyl linoleate, which indicates that the 18:2 component of mostly octadeca-5,11-dienoic acid in all the ester fractions.

The cyclopropane derivative of palmitic acid is the predominant fatty acid of the triglycerides in vegetative amoebae and represents a major constituent in the total lipids. This cyclopropane fatty acid content decreases with cell differentiation to the sorocarp stage; presumedly, the presence of the cyclopropane derivatives results from assimilation from the ingested *A. aerogenes*, and these fatty acids are replaced by others peculiar to *D. discoideum* during the course of differentiation. A major difference between the fatty acid distribution reported by Davidoff and Korn (4) and the one reported here is seen in the significant amounts of palmitic and stearic acids and in the absence of cyclopropane fatty acids in the earlier analysis. Again, this may reflect a difference in the diet, since the mutant strain used by the former authors was grown in the absence of bacterial lipid but in the presence of a medium which may have contained palmitic and stearic acids. A dependence of the fatty acid composition of amoebic lipids on fatty acids ingested from the medium has been abundantly demonstrated in a recent publication by Weeks (25). An axenic strain (Ax-2) grown in media containing linoleic or linolenic acids incorporates large amounts of these, converting them to fatty acids not normally found in *D. discoideum*, octadeca-5,9,12-trienoic acid and octadeca-5,9,12,15-tetraenoic acid, which again indicates the potency of the $\Delta 5$ desaturating system in this organism. Arachidonic acid is also incorporated but is not further desaturated. These polyunsaturated fatty acids appear to have little effect upon growth but cause alterations in development (25). It is interesting that the high level of cyclopropane fatty acids

found in amoebae lipids has no effect on growth or development of *D. discoideum*.

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Calcium Activation of Peanut Lipoxygenase[1]

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ABSTRACT

Peanut lipoxygenase isozyme 1 (pH optimum, 8.3) was strongly activated by 0.5-1.0 mM Ca^{++}, and the rate of activation was maximum when the ratio of substrate to Ca^{++} was ca. 2:1. Peanut lipoxygenase isozymes 2 and 3 (pH optima, 6.2) were activated by calcium but did not have an optimum level of activity. Calcium differentially activated peanut lipoxygenase causing the rate of pentane production to increase much more rapidly than the rate of oxygen consumed by the enzyme reaction. At pH 6.2, in the absence of calcium, the percentages of the hydroperoxide isomers produced by peanut lipoxygenase were 74.9% 13-hydroperoxy *cis*-9, *trans*-11-octadecadienoic acid (13 LOOH *cis-trans*), 2.6% 13-hydroperoxy *trans*-9, *trans*-11-octadecadienoic acid (13 LOOH *trans-trans*) and 22.5% 9-hydroperoxy 10, 12-octadecadienoic acid (9 LOOH). The presence of 1 mM Ca^{++} at pH 6.2 did not significantly affect the percentage distribution of the hydroperoxides produced. However, at pH 8.3, the percentage distribution of hydroperoxides produced was 45.2% 13 LOOH *cis-trans*, 10.9% 13 LOOH *trans-trans* and 43.9% 9 LOOH in the absence of Ca^{++} and 57.0% 13 LOOH *cis-trans*, 8.0% 13 LOOH *trans-trans* and 35.0% 9 LOOH in the presence of 1 mM Ca^{++}.

INTRODUCTION

Peanuts contain three lipoxygenase isozymes (E.C. 1.13.11.12), two of which have a pH optimum of 6.2 and one with a pH optimum of 8.3 (1). Peanut lipoxygenase isozyme 1 (pH 8.3) was shown to be progressively inhibited by increasing calcium concentrations, whereas isozymes 2 and 3 (pH 6.2) were activated by 40-400 μM calcium and inhibited by higher concentrations. These experiments were carried out with Tween 20 in the reaction mixture. However, Zimmerman and Synder (2) had shown soybean lipoxygenase 2 to be activated only in the absence of Tween 20, and Koch et al. (3) had shown Tween 20 to inhibit calcium activation at pH 7.5 and stimulate non-calcium activated navy bean lipoxygenase at pH 6.4. In light of these experiments, we decided to reexamine the calcium activation of peanut lipoxygenase.

Calcium activation of lipoxygenase from various sources is well documented and has been measured in several ways. Zimmerman and Synder (2) and Restrepo et al. (4) showed that calcium added to soybean lipoxygenase reactions increased oxygen consumption and conjugated diene formation and, hence, activated the enzyme reaction. Koch and coworkers (3) showed that calcium activates navy bean and soybean lipoxygenase by increasing conjugated diene formation. Christopher et al. (5) using gas liquid chromatography-mass spectrometry (GLC-MS) reported that soybean lipoxygenase 1 favors formation of the 13 positional hydroperoxide isomer under various conditions of pH and in the presence or absence of 0.55 mM Ca^{++}. At pH 7, soybean lipoxygenase 2 produced a mixture of the 13 and 9 positional hydroperoxide isomers in a 1:1 ratio in the presence of 0.55 mM Ca^{++} and a 38:62 ratio without calcium.

We proposed to determine the effect of calcium on three different facets of the peanut lipoxygenase reaction: the uptake of oxygen by the enzyme reaction, the effect on lipoxygenase-mediated pentane production, and the percentage distribution of the positional and geometrical hydroperoxide isomers produced.

EXPERIMENTAL PROCEDURES

Materials

Peanuts (*Arachis hypogaea* L. Var. NC-5) were obtained from the North Carolina Peanut Belt Research Station (Lewiston, NC); linoleic acid (Grade III, ca. 99% pure) was from the Sigma Chemical Co., St. Louis, MO; special enzyme grade ammonium sulfate and Tris were from Schwarz/Mann, Orangeburg, NY; and Sephadex G-25 and G-150 were from Pharmacia Fine Chemicals, Piscataway, NJ. All other chemicals used were reagent grade. Distilled, deionized water was used in the

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preparation of all buffers. Solvents used in hydroperoxide extraction and in high performance liquid chromatography (HPLC) were purified according to Pattee and Singleton (6).

Crude peanut extracts containing lipoxygenase activity were prepared from acetone powders of peanut seeds, and the partially purified lipoxygenase used in this study had been precipitated from crude extracts with ammonium sulfate and fractionated by Sephadex G-150 as previously described (1).

Enzyme Assays

Lipoxygenase activity was measured polarographically at 25 C with a Clark oxygen electrode. The 1.5 ml reaction vessel contained 1.8 mM linoleic acid (prepared immediately) before use with 0.1 M sodium borate buffer, pH 10) in either Tris-HCl (0.1 M, pH 8.2) or morpholino-ethane sulphonic acid buffer (0.1 M, pH 6.2). Lipoxygenase activity was calculated from the initial reaction rates, and the initial value for oxygen concentration was assumed to be 260 nmoles/ml.

Pentane Production

Pentane was produced in an apparatus that allows simultaneous measurement of oxygen consumption (7). The reaction conditions were identical to those used in the lipoxygenase assay except that the reaction volume was 3 ml. After 15 min of reaction time, 5 ml of gas was withdrawn from the reaction vessel and injected into a Varian Model 2700 gas chromatograph. A Chromasorb 102 column (6 ft x 1/8 in. stainless steel) programmed between 125 C and 200 C separated the volatile compounds produced. Peak areas were integrated with a Spectra-Physics Autolab I computing integrator.

Hydroperoxide Extraction and Analysis

Hydroperoxides formed by peanut lipoxygenase were extracted and chromatographed according to Pattee and Singleton (6). A Varian Model 8500 liquid chromatograph, with a Partisil 10 column, coupled to a Varian Techtron 635D spectrophotometer separated and monitored the different positional and geometrical hydroperoxide isomers formed. The isomers were identified as reported; peak areas were integrated as described above.

RESULTS AND DISCUSSION

Calcium Effect on Oxygen Consumption by Lipoxygenase

In the absence of Tween 20, peanut lipoxygenase was activated by calcium at either pH

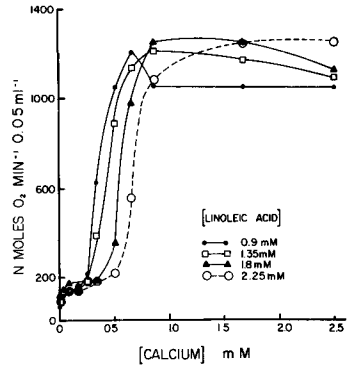

FIG. 1. Effect of Ca^{++} and various substrate concentrations on the activity of crude peanut lipoxygenase measured polarographically at pH 8.3.

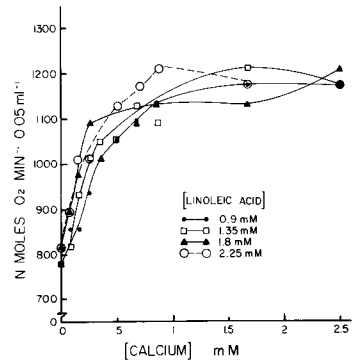

FIG. 2. Effect of Ca^{++} and various substrate concentrations on the activity of crude peanut lipoxygenase measured polarographicaly at pH 6.2.

6.2 and 8.3 (the pH optima of the peanut isozymes). At pH 8.3 (Fig. 1), the calcium concentration for maximum reaction rate varied with the substrate concentration, with the highest reaction rate occurring when the ratio of substrate to calcium is ca. 2:1. This relationship suggests that activation is maximum when most of the substrate is converted to calcium linoleate, which consists of 2 lipid moieties per Ca^{++}. Zimmerman and Synder (2) suggested that calcium interacts with the substrate to make it more accessible to the lipoxygenase 2 isozyme of soybeans. In our work the action of peanut isozyme 1 appeared to act similarly to that of the lipoxygenase 2 isozyme of soybeans; however, activation of the soybean isozyme was maximum when the calcium concentration was about equimolar to the substrate concentration (4).

At pH 6.2 (Fig. 2), the enzyme was activated by various concentrations of calcium, but activation was not as high as at pH 8.3. At pH

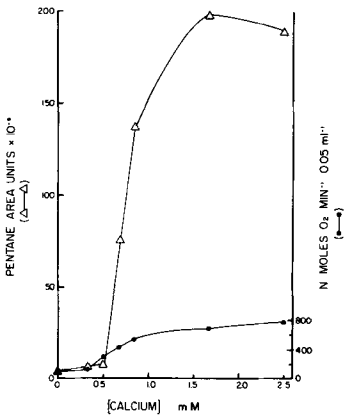

FIG. 3. Activity of partially purified peanut lipoxygenase measured polarographically (●—●) and by pentane production (△—△) at pH 8.3 in relation to various Ca^{++} concentrations. Data is graphed to scale by plotting points on a percent increase basis over the control (no calcium). The concentration of linoleic acid was 1.8 mM.

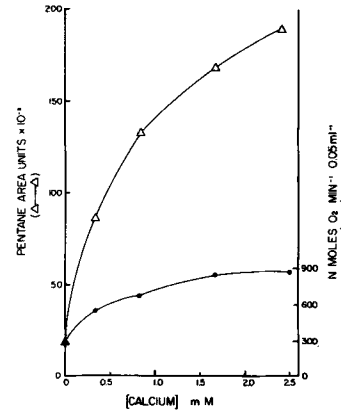

FIG. 4. Activity of partially purified peanut lipoxygenase measured polarographically (●—●) and by pentane production (△—△) at pH 6.2 in relation to various Ca^{++} concentrations. Data is graphed to scale by plotting points on a percent increase basis over the control (no calcium). The concentration of linoleic acid was 1.8 mM.

6.2 and constant levels of calcium, enzyme activity generally increased with increased substrate concentration. Increasing the calcium concentration only appeared to increase the rate of activity relative to the different substrate concentrations up to a point where activity no longer increased. Although calcium stimulated lipoxygenase activity at pH 6.2, the presence of calcium linoleate as the substrate did not appear to be as critical as at pH 8.3. Both crude and partially purified enzyme preparations yielded calcium response curves similar to Figures 1 and 2, although the partially purified preparations, with lower concentrations of calcium absorbing protein, yielded slightly higher activation levels.

Calcium Effects on Pentane Production

Pentane and hexanal are the major volatile components of the peanut lipoxygenase reaction (8). The effect of calcium on the pentane producing capability of peanut lipoxygenase is illustrated in Figures 3 and 4. At pH 8.3 (Fig. 3), enzyme activity (measured by the consumption of oxygen by the enzyme reaction) and pentane production increased rapidly between 0.5 and 0.833 mM Ca^{++}, but the rate of pentane production was much more rapid than the rate of oxygen consumption by the enzyme. At pH 6.2 (Fig. 4), both oxygen consumption and pentane production increased, and again the rate of production of pentane increased much faster than the rate of consumption of oxygen although not as fast as at pH 8.3.

Johns et al. (9) and Pattee et al. (8) were unable to separate pentane production from lipoxygenase activity even when the enzyme had been purified by isoelectric focusing with a 0.5 pH gradient or by gel filtration and ion exchange chromatograph. Smith and Lands (10) proposed a kinetic system for lipoxygenase action involving two binding sites, one for the substrate (S) only, and one that bound the product (P) hydroperoxide and, possibly the substrate. Johns et al. (9) indicated that increasing substrate concentrations decreased pentane production, possibly because of increased substrate binding at the P sites. Since

TABLE II

Changes (Δ) in the Percentage Distribution and Yields of Geometric Hydroperoxide Isomers Obtained at pH 6.2 and pH 8.3 in the Presence of 1 mM Ca^{++} over the control (no calcium)

	Δ13 LOOH *cis-trans*		Δ13 LOOH *trans-trans*		Δ9 LOOH		
pH	% Composition	mg LOOH	% Composition	mg LOOH	% Composition	mg LOOH	Total mg LOOH
6.2	77.6	11.6	4.8	0.7	17.6	2.6	14.9
8.3	74.4	3.9	3.7	0.2	21.9	1.2	5.3

pentane is produced by cleavage of the 13 positional hydroperoxide isomer at the P binding site, our results indicate that increasing levels of calcium may affect product cleavage (pentane production) at the P binding site more than oxygen consumption at the S binding site. Thus, in addition to increasing substrate affinity for the enzyme, calcium may also enhance hydroperoxide cleavage so that the reaction equilibrium is shifted in favor of pentane formation.

Calcium Effects on Hydroperoxide Ratios

Pattee and Singleton (6) using HPLC identified 3 positional and geometrical hydroperoxide isomers produced by peanut lipoxygenase: 13-hydroperoxy *cis*-9, *trans*-11-octadecadienoic acid (13 LOOH *cis-trans*), 13-hydroperoxy *trans*-9, *trans*-11-octadecadienoic acid (13 LOOH *trans-trans*) and 9-hydroperoxy 10, 12-octadecadienoic acid (9 LOOH). Subsequent work has shown this peak to be a mixture of the *trans-cis* and *trans-trans* isomers of 9-LOOH (Pattee and Singleton, unpublished data). Table I shows the percentage distribution of each component produced in our experiments under various conditions of pH and calcium concentration. All values shown are the means of at least three individual replications corrected for initial hydroperoxide levels arising from autoxidation of the substrate. All replicates had equal amounts of enzyme activity. At either pH, there was an increase in the amount of hydroperoxide produced in the presence of 1 mM calcium chloride. The percentage increase in the amount of the 13 LOOH *cis-trans* isomer produced in the presence of 1 mM Ca^{++} was large at pH 8.3, but only very slight at pH 6.2. The lower total amount of hydroperoxides produced at pH 8.3 than at 6.2 indicates that the peanut lipoxygenase isozyme 1 is much less active than isozymes 2 and 3, especially when sodium linoleate is used as the substrate.

If the current hypothesis is correct that only the *cis-trans* hydroperoxide isomers are formed by the enzymatic action of lipoxygenase, then the large increase in the percentage of the 13 LOOH *cis-trans* isomer produced at pH 8.3 as a result of added Ca^{++} may be a reflection of this fact. The change in the percentage distribution of the geometric hydroperoxide isomers obtained in the presence of 1 mM Ca^{++} over the control reaction (Table II) shows that when calcium was added to the enzyme reaction, the major hydroperoxide formed at pH 8.3 was the 13 LOOH *cis-trans* isomer and that the percentage distribution of the hydroperoxides formed at pH 8.3 was similar to that at pH 6.2.

TABLE I

Percentage Distribution of the Geometric Hydroperoxide Isomers Obtained by Varying pH and Calcium Concentration[a]

pH	Ca^{++} [1 mM]	13 LOOH *cis-trans*		13 LOOH *trans-trans*		9 LOOH		Total mg LOOH
		% Composition	mg LOOH	% Composition	mg LOOH	% Composition	mg LOOH	
6.2	−	74.9	31.3	2.6	1.1	22.5	9.4	41.8
6.2	+	75.6	42.9	3.2	1.8	21.2	12.0	56.7
8.3	−	42.2	3.5	10.9	0.8	43.9	3.4	7.7
8.3	+	57.0	7.4	8.0	1.0	35.0	4.6	13.0

[a]The reaction was saturated with oxygen at 25 C, extracted at 4 C, and the calcium concentration was either 1 mM or zero.

The data allows the speculation that a second reaction may take place simultaneously to the Ca^{++} stimulated reaction and produces hydroperoxides in about a 1:1 ratio of 13 and 9 positional isomers at pH 8.3.

Since hydroperoxide formation and oxygen consumption should be stimulated by Ca^{++} at the same rate, one should not attempt to relate the marked Ca^{++} induced difference between the rates of pentane production and oxygen uptake (Figs. 3 and 4) with the increase in the total percentage distribution of the 13 positional hydroperoxide isomers in the presence of calcium. Also, the sensitivity of the pentane analysis is several orders or magnitude greater than that of the assay for hydroperoxides. Therefore, an increase in the rate of pentane production would not significantly effect the ratio of 13:9 positional hydroperoxide isomers even when this production is stimulated by calcium.

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The Effect of Dietary Fats on the Plasma Lipid Composition of Sheep

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ABSTRACT

This study reports on the plasma lipid compositions of sheep fed either a control diet (C), a control diet supplemented with tallow (A) or polyunsaturated fatty acid (B) that had been protected against hydrolysis and hydrogenation in the rumen, or a control diet supplemented with maize oil (D). Diet B considerably increased the 18:2 content of all the major plasma lipid fractions. Although the feeding of diet D also resulted in an increase in the 18:2 contents within the cholesteryl ester, unesterified fatty acid, and phospholipid fractions, the increases were considerably less than those observed with diet B; the levels of 18:2 within the triglyceride fraction remained similar to that for the sheep which received the control diet. The effect of feeding diet A was confined solely to the triglyceride fraction where the concentrations of 16:0 and 18:1 were increased. The lipoproteins of the plasma were separated into very low density lipoproteins ($d<1.006$), low density lipoproteins ($1.006<d<1.063$), and high density lipoproteins ($1.063<d<1.21$), and the distribution of the major lipids between these lipoprotein fractions was investigated. Diet B increased considerably the proportion of triglyceride found in association with the very low density fraction and the concentrations of 18:2 within all the lipoprotein fractions; these increases in the concentrations of 18:2 were not confined to any particular lipid fraction of the lipoproteins. In contrast, the increases in the concentrations of 18:2 produced as a result of feeding diet D were confined to the low and high density lipoproteins. The effect of feeding diet A was confined to fatty acid changes within the triglycerides of the low and very low density lipoproteins.

INTRODUCTION

A great deal is now known about the regulatory function exerted by the microbial population within the forestomach of the ruminant animal on the utilization of dietary lipids. As a result of the extensive hydrolysis, isomerization, and hydrogenation of dietary lipids that occur within the rumen (1,2), the normally large amounts of polyunsaturated fatty acids (PUFA) ingested by the ruminant animal are saturated and little of the dietary PUFA is available for absorption from the small intestine. Attempts to overcome this by increasing the PUFA content of the diet are largely ineffectual and may in fact have far reaching secondary effects on other aspects of ruminant metabolism (3). Recently, however, techniques for protecting the PUFA of the diet from rumen activity by a process of encapsulation within a coating of formaldehyde-treated protein have been developed (4-7). By this means, the PUFA content of the carcass and milk of ruminant animals can be greatly elevated. However, the mode of utilization and metabolism between absorption and final incorporation into the tissues has largely been neglected. In the present investigations, therefore, the changes in the concentration of plasma lipids and lipoproteins, together with their fatty acid compositions, have been studied in sheep fed control diets or diets containing unprotected maize oil, or tallow or a mixture of sunflower seedoil and soybean oil protected by treatment with formaldehyde treated protein.

MATERIALS AND METHODS

Four groups of 6-month-old Cheviot sheep, five animals per group, were fed either a control diet (diet C) consisting of 500 g of hay and 300 g of a cereal concentrate mixture per day or diets in which a proportion of the concentrate had been replaced by "protected" tallow (diet A), "protected" PUFA composed of ca. 70% sunflowerseed oil and 30% soybean oil (diet B) or maize oil (diet D). In the supplements of PUFA and tallow, protection against hydrogenation in the rumen was provided by a formaldehyde-treated protein coat (5); these dietary supplements were donated by Alta Lipids U.K. Ltd. (London, England). The total fat intake of each of the sheep on the control diet was 32 g per day (20 g from the hay and 12 g from the cereal mixture) and the fat intake of the sheep

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receiving the fat supplemented diets was 60 g per day (31 g from the fat supplement, 9 g from the cereal mixture and 20 g from the hay). The fatty acid compositions of the dietary constituents were as detailed previously (8). The animals were housed in individual pens; water was available ad libitum. All the animals were maintained on the diets for 6 wk.

Blood samples (ca. 100 ml) were obtained from the jugular vein of each animal at 10.00 hr by means of heparinized Vacutainer tubes (Becton-Dickinson, Rutherford, NJ) that had been chilled to 4 C. The lipids were extracted from the plasma by the method of Nelson and Freeman (9) and the plasma lipids separated by thin layer chromatography. The fatty acid compositions of the lipid classes were determined by gas liquid chromatography of methyl ester derivatives on packed columns of EGSS-X, and the absolute concentrations of these lipid classes determined by the addition to each fraction of a known amount of n-heptadecanoic acid as an internal standard (10). Free cholesterol concentrations were determined by gas chromatography on packed columns of 3% OV17 using an internal standard of 5-α cholestane (11). Serum lipoproteins were separated by ultracentrifugation according to the method of Lindgren et al. (12). Fractions collected were very low density lipoproteins (VLDL), $d<1.006$; low density lipoproteins (LDL) $1.006<d<1.063$; high density lipoproteins (HDL), $1.063<d<1.21$; the remainder of the plasma lipoproteins ($d>1.21$) contained unesterified fatty acids and constituted only about 2% of the total plasma lipids. VLDL fractions were washed by resuspension in physiological saline followed by recentrifugation under the conditions of the original separation. All other lipoprotein fractions were obtained without washing. Electrophoretic separations of the lipoprotein fractions on cellulose acetate (13) indicated little contamination of lipoprotein classes between fractions.

RESULTS

No significant effects on the concentrations and distributions of the major plasma lipids were observed as a result of the various dietary treatments. The mean concentration of the total lipid within the plasma of the sheep was 125 mg per 100 ml and had the following percentage composition: cholesteryl esters, 42; free cholesterol, 8; triglycerides, 7; unesterified fatty acids, 3; phospholipids, 40.

The fatty acid compositions of the cholesteryl ester, triglyceride, unesterified fatty acid and phospholipid fractions in the plasma of the sheep after the various dietary treatments are given in Table I. In the sheep which received diet C, appreciable concentrations of PUFA were present within the cholesteryl ester and phospholipid fractions and only low concentrations present within the triglyceride and unesterified fatty acid fractions. By far, the most significant effects on the fatty acid compositions of the various plasma lipids resulted from the feeding of diet B. When this diet was fed, the concentrations of 18:2 in all the plasma lipid components were significantly increased ($P<0.001$) above the corresponding levels found in the lipids of the plasma from the sheep which received either diets A or C. Feeding diet D to the sheep also resulted in a small but significant ($P<0.05$) increase in the concentrations of 18:2 within the cholesteryl ester, phospholipid and unesterified fatty acid fractions of the plasma. However, in these sheep the levels of 18:2 in the triglyceride fraction remained similar to those for the plasma triglycerides from the sheep that received either diets A or C. In spite of the increased concentrations of 18:2 in the plasma lipids achieved by feeding diet D, in every instance the levels were significantly lower ($P<0.05$) than those achieved by feeding diet B. These increases in the plasma concentrations of 18:2 which occurred as a result of feeding the polyunsaturated fatty acid diets were counterbalanced mainly by a decrease in the concentration of 18:1. In the sheep that received diet A, the changes that occurred in the plasma fatty acids were confined to the triglyceride fraction where, compared with the levels found in the plasma from the sheep fed diet C, the concentrations of both 16:0 and 18:1 were significantly increased ($P<0.01$ and $P<0.05$, respectively) at the expense of a decreased concentration ($P<0.01$) of 18:0.

The distribution of the total lipid concentrations between the VLDL, LDL, and HDL fractions of the plasma, together with the distribution of the major plasma lipid components between the lipoprotein classes, is shown in Table II. It can be seen that in each group of sheep the HDL fraction was the major lipid bearing class accounting for between 60-75% of the total lipid circulating within the plasma. Most of the remaining lipid was associated with the LDL fraction as the VLDL fraction accounted for only 5-10% of the total plasma lipid concentration. No obvious dietary effects on the distribution of the total plasma lipids between the lipoprotein fractions could be discerned. The distribution of the cholesteryl esters, phospholipids, and the unesterified fatty acids between the lipoprotein fractions paral-

TABLE I

The Fatty Acid Compositions (Major Components, Weight Percentages of the Total) of the Triglycerides, Unesterified Fatty Acid, Cholesteryl Ester and Phospholipid Fractions of the Sheep Plasma Lipids

Triglycerides	Diet A Protected tallow	Diet B Protected PUFA[a]	Diet C Control	Diet D Maize oil	SE ±	Significant effects
16:0	32.7	17.0	22.2	21.1	1.35	AVB[b], AVC[c], AVD[b]
16:1	4.7	2.6	3.7	3.4	0.62	
18:0	22.7	36.9	40.1	40.5	2.87	AVB[d], AVC[c], AVD[d]
18:1	31.6	18.9	25.8	25.4	1.58	AVB[c], AVD[d], AVC[d], BVC[d]
18:2	5.9	22.6	5.9	8.1	2.56	AVB[c], BVC[c], BVD[d]
18:3	2.4	2.2	2.6	1.4	0.80	
Cholesteryl esters						
16:0	11.2	8.3	11.5	10.1	0.82	
16:1	5.4	2.0	6.3	3.0	0.71	AVB[d], BVC[c], CVD[d]
18:0	2.6	2.8	2.7	3.7	0.30	
18:1	41.7	16.4	38.9	24.7	1.96	AVB[b], AVD[b], BVC[b], BVD[d], CVD[c]
18:2	33.5	68.6	37.9	52.6	3.42	AVB[b], AVD[c], BVC[b], BVD[d], CVD[d]
18:3	2.6	2.0	2.7	3.7	0.75	
20:4	3.5	<1.0	<1.0	2.6	0.31	

Unesterified fatty acids	Diet A Protected tallow	Diet B Protected PUFA	Diet C Control	Diet D Maize oil	SE ±	Significant effects
16:0	22.6	17.4	21.4	18.4	2.25	
16:1	2.7	2.3	2.1	2.2	0.25	
18:0	35.5	35.2	39.6	30.7	2.39	
18:1	32.0	30.2	31.5	36.3	2.37	
18:2	5.6	13.3	4.7	8.9	0.82	AVB[b], AVD[d], BVC[b], BVD[d], CVD[d]
18:3	1.6	1.6	0.9	2.2	0.65	
Phospholipids						
16:0	18.6	12.8	16.1	15.2	0.36	AVB[c], AVD[d], BVC[d]
16:1	1.3	0.7	1.0	0.9	0.16	AVB[d]
18:0	24.5	26.7	23.6	24.8	1.32	
18:1	26.7	9.8	26.1	18.7	1.35	AVB[b], AVD[c], BVC[b], BVD[c], CVD[d]
18:2	18.0	38.1	21.8	31.2	1.82	AVB[b], AVD[c], BVC[b], BVD[d], CVD[d]
18:3	1.4	2.3	1.6	1.3	1.04	
20:4	9.6	9.8	9.2	7.6	1.19	

[a]PUFA = polyunsaturated fatty acid.

[b]Level of significance between groups is $P<0.001$.

[c]Level of significance between groups is $P<0.01$.

[d]Level of significance between groups is $P<0.05$.

TABLE II

Distribution of Major Lipid Classes between the Lipoprotein Fractions of the Sheep Plasma[a]

	Diet A Protected tallow			Diet B Protected PUFA		
	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)
Total	7.6 (5.0-10.8)	31.8 (25.0-43.8)	60.5 (45.5-68.2)	7.9 (5.0-10.1)	19.8 (12.7-31.0)	72.4 (60.7-79.2)
Cholesteryl esters	2.4 (1.0-3.8)	30.4 (25.4-32.5)	67.7 (65.0-69.6)	2.7 (1.4-4.8)	22.6 (11.6-31.7)	74.7 (66.3-87.1)
Triglycerides	31.6 (29.7-32.4)	62.8 (62.5-64.1)	6.2 (4.0-7.8)	71.1 (70.9-71.3)	25.5 (23.9-29.1)	3.9 (1.0-6.9)
Unesterified[b] fatty acids	-	-	-	-	31.0 (24.9-36.8)	68.9 (63.3-75.0)
Free cholesterol	-	55.3 (44.0-66.2)	44.8 (35.0-60.7)	-	37.1 (28.2-53.9)	62.9 (47.2-72.9)
Phospholipids	2.9 (1.0-5.7)	23.2 (19.5-30.2)	74.2 (64.0-80.2)	1.8 (1.0-2.3)	16.5 (11.9-25.3)	82.1 (72.5-87.8)
	Diet C Control			Diet D Maize oil		
Total	8.3 (6.5-11.2)	32.8 (19.8-42.9)	58.9 (50.2-59.4)	3.6 (2.1-5.1)	24.9 (13.0-35.3)	71.5 (60.0-84.0)
Cholesteryl esters	3.5 (1.3-8.9)	30.5 (25.8-38.1)	66.1 (58.3-72.9)	2.7 (2.4-3.0)	22.3 (13.4-27.6)	74.9 (70.6-83.2)
Triglycerides	11.2 (9.2-20.6)	55.7 (54.0-57.8)	11.2 (9.2-20.6)	19.7 (8.2-30.0)	65.1 (52.5-81.7)	15.2 (8.2-20.3)
Unesterified fatty acids	-	-	-	-	26.7 (14.0-45.4)	72.1 (55.0-82.5)
Free cholesterol	-	49.0 (41.1-63.6)	51.0 (35.7-61.4)	-	50.2 (46.3-61.9)	49.7 (43.0-69.0)
Phospholipids	3.2 (1.4-4.4)	27.4 (21.8-36.5)	69.5 (59.2-76.8)	2.1 (1.0-4.5)	18.0 (11.1-30.0)	80.7 (68,0-90.2)

[a]Mean values (weight percentages of total) and ranges for five animals are given in each instance. Abbreviations: PUFA = polyunsaturated fatty acids; VLDL = very low density lipoprotein; LDL = low density lipoprotein; HDL = high density lipoprotein.

[b]Unesterified fatty acids were mainly associated with d>1.21 fraction (see text).

leled that of the total lipid with some 60-80% of each being associated with the HDL fraction, about 20-30% with the LDL fraction, and less than 10% with the VLDL fraction; free cholesterol was more evenly distributed between the HDL and LDL fractions. In contrast with the other major plasma lipid fractions, the triglycerides were associated largely (>85%) with the VLDL and LDL fractions. A marked dietary effect could discerned in the distribution of the triglycerides between the lipoprotein fractions. Thus, whereas in the sheep which received diets A, C, or D, the LDL and VLDL fractions accounted for some 55-65% and 10-30%, respectively, of the total plasma triglycerides, in the case of the sheep given diet B the amount of triglyceride associated with the VLDL fraction increased to ca. 70% of the total plasma triglyceride and there was a concomittant decrease in the proportion of the triglyceride associated with the LDL fraction. Specific compensatory changes in the distributions of the cholesteryl ester, phospholipid, and free cholesterol fractions between the lipoprotein fractions were not obvious.

Table III shows the lipid compositions of each of the lipoprotein classes in the plasma of the sheep. The lipid compositions of the HDL and LDL fractions showed marked similarities; in both, the cholesteryl esters and phospholipids constituted the major lipid components and together accounted for more than 70% of the total lipid present in each case. However, in the LDL fraction, the proportions of both triglyceride and free cholesterol were significantly higher than within the HDL fraction. Although the VLDL fraction also contained a relatively high proportion of cholesteryl esters and phospholipid, they were characterized principally by high proportions of triglyceride. There were no obvious dietary effects upon the lipid compositions of the lipoprotein fractions in the animals given diets A and D. On the other hand, in the animals fed diet B, the proportion of triglyceride within the VLDL fraction underwent a considerable increase and there was a compensatory decrease in the proportion of cholesteryl esters.

The distributions of fatty acids within the cholesteryl ester, phospholipid and triglyceride fractions of the plasma lipoproteins are listed in Table IV. It is clear from the fatty acid patterns of the sheep given diet C that pronounced differences existed in the degree of saturation between the high and low density lipoprotein fractions as the proportion of 18:2 in the HDL fraction exceeded that of LDL which in turn exceeded that in VLDL; there were compensatory changes in the proportions of 16:0 and 18:0. Feeding diet B considerably increased the proportions of 18:2 and decreased the proportions of 16:0 and 18:1 in all three lipoprotein fractions compared with the animals given diet C; the feeding of diet D increased the concentrations of 18:2 within the HDL and LDL fractions above those found in the animals fed diet C. In all instances, the effects of feeding diet B on 18:2 concentrations were considerably greater than were the effects of feeding diet D. The effect of feeding diet A on the total fatty acid concentrations was confined to the VLDL fraction where, compared with the animals given diet C, the proportions of 18:1 and possibly 16:0 were increased at the expense of 18:0.

In the sheep fed diet C, it can be seen that the cholesteryl esters of the HDL fraction were characterized by particularly high concentrations of 18:2 and 18:3 which together accounted for over 80% of the total fatty acids present. Within the lower density fractions and in particular the VLDL fraction, the proportion of unsaturated fatty acids in the cholesteryl esters was very much lower and there were concomittant increases in the proportions of 16:0 and 18:0. Any 20:4 present in the cholesteryl esters was confined to the HDL fraction. Unlike the cholesteryl esters, the distributions of fatty acids within the phospholipids of the HDL, LDL, and VLDL fractions were very similar with about an equal distribution between saturates and unsaturates except that 20:4 again appeared to be preferentially associated with the HDL fraction. Although the triglycerides in all the lipoprotein fractions contained only low concentrations of 18:2, in common with the cholesteryl esters, there appeared to be a preferential association of 18:2 with the HDL fraction. In general, however, the triglycerides showed little difference in composition between the lipoprotein fractions.

The effect of feeding diet B on the 18:2 concentrations within the lipoprotein fractions was not confined to any particular lipid or lipoprotein fraction as considerable increases in the concentrations of 18:2 occurred throughout. In comparison, the effects of feeding diet D on the 18:2 concentrations were not only considerably smaller than those produced by feeding diet B but, in the case of both the phospholipids and triglycerides, were confined mainly to the HDL fraction. Thus, whereas in the triglycerides circulating in association with the VLDL fraction, feeding diet B increased the concentrations of 18:2 from ca. 2% up to 23%, feeding diet D increased the level of 18:2 up to only ca. 9%. While the increases in the proportions of 18:2 within the cholesteryl esters and

TABLE III

Lipid Composition of the Lipoprotein Fractions of the Sheep Plasma[a]

	Diet A Protected tallow			Diet B Protected PUFA		
	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)
Cholesteryl esters	36.2 (33.5-37.9)	49.8 (46.2-52.9)	55.1 (49.2-55.9)	18.3 (7.3-20.6)	45.7 (38.5-53.2)	51.1 (41.0-59.7)
Triglycerides	45.2 (44.3-49.4)	13.5 (17.8-11.3)	<1.0	66.4 (51.5-76.6)	9.1 (4.7-9.5)	2.6 (1.0-7.0)
Unesterified fatty acids	-	-	-	-	2.8 (2.4-2.9)	4.0 (2.5-5.8)
Free cholesterol	-	18.5 (14.3-22.2)	7.8 (6.1-11.7)	-	16.7 (9.1-27.0)	12.0 (5.0-17.6)
Phospholipids	18.6 (16.3-19.9)	18.0 (17.4-19.0)	36.4 (34.8-36.0)	15.9 (9.6-27.2)	25.8 (21.0-28.9)	30.2 (23.3-35.8)
	Diet C Control			Diet D Maize oil		
Cholesteryl esters	14.0 (9.0-17.1)	43.9 (40.4-50.0)	48.7 (48.2-49.7)	36.5 (25.3-46.5)	37.7 (26.5-46.0)	43.4 (37.6-47.9)
Triglycerides	59.5 (54.6-67.0)	12.7 (10.7-14.2)	1.3 (2.2-1.0)	44.2 (35.7-49.9)	16.6 (8.7-22.7)	1.9 (1.2-3.9)
Unesterified fatty acids	-	-	-	-	3.1 (1.1-6.5)	4.2 (1.3-7.7)
Free cholesterol	-	15.6 (13.1-17.4)	12.4 (8.2-19.8)	-	19.5 (18.0-24.1)	11.6 (7.3-13.8)
Phospholipids	26.6 (14.9-32.2)	28.2 (26.2-29.8)	38.1 (35.7-39.9)	19.4 (18.0-27.6)	23.1 (20.4-25.1)	38.9 (32.6-45.5)

[a]Mean values (weight percentages of total) and ranges for five animals are given in each instance. For identification of abbreviations see Table II.

phospholipids were accompanied by decreased concentrations of 18:1, in the triglycerides, changes in the proportions of 16:0 and 18:0 were also apparent. The effect of feeding diet A was confined to the fatty acids contained within the triglycerides of the LDL and VLDL fractions where the proportions of 16:0 18:1 were increased and the proportion of 18:0 decreased.

DISCUSSION

The concentrations and fatty acid compositions of the various lipid fractions in the plasma of the sheep in the present experiment were similar to those reported previously for sheep and ruminant animals in general (14-17). Thus, the cholesteryl esters and phospholipids comprised the principal components (ca. 75-80% of the total lipids present) and were accompanied by considerably smaller quantities of triglycerides, unesterified fatty acids, and free cholesterol. The 18:2, which accounted for a considerably proportion of the total fatty acids present in the plasma, was found predominantly in the cholesteryl ester and phospholipid fractions.

Only limited information (18,19) is available on the composition of ovine plasma lipoproteins in comparison to that of cattle or of nonruminant species. A preferential association of the plasma lipid with the HDL fraction would appear to be general for ruminant animals (18-24) in marked contrast to the distribution of the plasma lipids between the various lipoprotein fractions in man where the HDL ($d>1.063$) accounts for ca. 23% and the LDL ($d<1.063$) ca. 77% of the total circulatory lipid (25-27). The plasma of the ruminant animal is also further characterized by the extremely low concentrations of lipid within the VLDL and chylomicron ($d<1.006$) fractions (21,23,24). In the ruminant animal, the low concentration of lipoproteins and chylomicrons with a density <1.006 within the plasma has been correlated with the continuous nature of the lipid absorption processes within the gastrointestinal tract as opposed to the intermittent fluxes which predominate in other species (23,28). The overall distribution of the major lipid classes within and between each plasma lipoprotein fraction in the sheep in the present experiment was broadly similar to that which has been found by others for ruminant (18,20,24) and nonruminant animals (25,27,29,30).

In the present work, extensive increases in the PUFA contents of the plasma lipids were obtained by circumventing the biohydrogenative capacity of the rumen via the use of the "protected" PUFA diet. In the sheep receiving diet D, the increase in the concentration of PUFA achieved within the plasma was associated with those fractions (the cholesteryl esters and phospholipids) which already carried the major part of the circulatory PUFA. The ability to increase the PUFA content of these fractions even further under these conditions as opposed to the triglyceride fraction where the concentration of PUFA remains relatively low has been explained in terms of the transitory nature of the supply of PUFA from the intestine and the differential rates for the turnover and hydrolysis of the various plasma lipid components (31,32). In contrast, by feeding diet B, these effects were largely overcome and by creating a pattern of lipid metabolism similar to that which would be expected to operate within the nonruminant animal, a permanently high level of 18:2 was achieved within the plasma triglycerides. For example, it was possible that in the latter instance, higher proportions of monoglycerides containing the 18:2 were available within the intestine for synthesis into triglycerides via the monoglyceride pathway. The effects of diet A, on the other hand, on plasma fatty acid composition were confined solely to the triglyceride fraction. In view of the known discrimination of the cholesteryl esters and phospholipids towards the metabolism of PUFA, this disproportionate effect on the fatty acids of the plasma triglycerides was to be expected.

The differences in the proportions of 18:2 present within the total fatty acids of the high and low density lipoprotein fractions observed in the plasma of the sheep were accounted for almost entirely by the differences in the level of 18:2 present within the cholesteryl esters associated with each fraction. In bovine plasma, the partition of 18:2 between the cholesteryl esters of the high and low density lipoprotein fractions has been observed to be even more pronounced (20,21,33). In contrast to these results for ruminant plasma, the cholesteryl esters of human plasma show a great deal of similarity in their fatty acid composition in the different lipoprotein classes (34). Such extreme variation in the fatty acid compositions of the cholesteryl ester associated with the different lipoprotein fractions between animals species has been cited to correspond with the different biosynthetic origins of the plasma cholesteryl esters, i.e. whether they arise from within the plasma via the lecithin-cholesterol-acyltransferase (LCAT) system or from hepatic synthesis (35-38).

Although the present investigations showed that there were no dietary effects on the overall

TABLE IV

The Fatty Acid Compositions of the Cholesteryl Ester, Phospholipid and Triglyceride Fractions within the Lipoprotein Fractions of the Sheep Plasma[a]

	Diet A Protected tallow			Diet B Protected PUFA		
Cholesteryl esters	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)	VLDL (d<1.006)	LDL (d>1.006, <1.063)	HDL (d>1.063, <1.21)
16:0	25.5 (15.1-28.5)	14.1 (13.0-17.1)	11.3 (10.3-12.4)	18.7 (18.3-19.0)	10.4 (9.8-15.8)	8.8 (7.0-11.8)
16:1	9.2 (7.1-11.8)	5.5 (4.2-6.4)	4.2 (3.4-5.0)	7.9 (7.4-12.5)	2.8 (2.1-3.8)	1.2 (1.0-2.0)
18:0	12.6 (7.4-15.7)	6.2 (3.0-9.1)	1.0 (0.3-1.9)	20.8 (19.4-23.1)	4.9 (4.4-5.4)	2.3 (1.0-4.8)
18:1	39.3 (32.1-47.4)	39.7 (37.8-41.1)	40.5 (38.9-42.7)	19.7 (15.0-24.7)	23.0 (17.6-27.1)	11.6 (7.4-19.1)
18:2	13.8 (7.6-20.7)	30.9 (28.2-33.7)	39.6 (30.4-41.5)	28.1 (20.2-37.5)	60.2 (53.1-65.9)	70.1 (51.0-78.5)
20:4	<1.0	2.8 (1.0-4.6)	3.3 (1.0-5.1)	<1.0	<1.0	3.7 (2.2-4.2)
Phospholipids						
16:0	29.5 (24.5-32.5)	23.4 (21.0-24.6)	18.5 (16.7-19.7)	19.3 (16.2-21.8)	16.4 (15.0-18.3)	12.9 (11.7-14.4)
16:1	2.9 (2.5-3.0)	0.6 (0.5-1.0)	0.9 (0.5-1.0)	1.7 (1.9-2.9)	<1.0	<1.0
18:0	18.9 (16.2-21.8)	26.1 (23.6-30.7)	25.9 (23.8-27.0)	25.6 (23.3-26.9)	28.2 (27.0-30.0)	26.5 (23.9-27.8)
18:1	27.8 (25.3-31.3)	27.3 (24.6-29.7)	29.2 (26.1-34.0)	20.6 (15.0-25.8)	9.4 (9.0-10.7)	10.0 (7.8-13.0)
18:2	19.4 (16.4-23.5)	16.2 (14.4-20.4)	17.5 (15.1-18.4)	29.5 (28.0-31.8)	37.8 (35.7-40.9)	39.1 (32.3-43.4)
20:4	<1.0	6.3 (4.7-7.1)	7.7 (6.3-10.0)	1.9 (2.2-3.6)	7.9 (6.0-9.0)	11.2 (7.7-13.7)
Triglycerides						
16:0	30.2 (27.6-32.0)	32.7 (30.2-34.2)	-	16.5 (15.1-18.3)	14.2 (11.8-16.3)	19.8 (18.7-24.1)
16:1	2.6 (2.4-2.7)	2.8 (1.8-3.3)	-	2.0 (0.7-3.2)	1.9 (1.2-2.3)	7.1 (4.1-9.1)
18:0	18.4 (14.3-24.5)	31.4 (30.2-32.2)	-	34.2 (30.2-39.0)	29.4 (15.5-37.9)	21.2 (15.0-25.0)
18:1	37.4 (34.3-42.6)	27.3 (26.7-28.2)	-	19.8 (17.9-22.0)	20.4 (17.8-26.4)	18.5 (14.1-21.0)
18:2	9.0 (4.8-14.2)	5.2 (3.4-7.4)		23.4 (15.0-29.5)	34.7 (28.2-43.2)	25.2 (15.5-35.1)

Cholesteryl esters	Diet C Control			Diet D Maize oil		
16:0	22.9 (20.1-24.8)	13.9 (11.0-14.3)	10.1 (8.9-11.2)	26.2 (21.8-29.7)	12.1 (10.0-13.1)	8.9 (7.9-10.9)
16:1	6.1 (4.8-7.3)	8.3 (6.2-9.9)	4.4 (4.0-5.1)	14.8 (12.0-15.1)	4.4 (3.1-7.2)	2.1 (1.5-2.4)
18:0	31.4 (26.5-36.8)	7.3 (4.4-11.7)	1.5 (0.7-2.8)	12.7 (7.5-18.9)	8.0 (5.2-9.9)	1.5 (1.2-2.0)
18:1	24.3 (23.4-26.1)	41.7 (36.0-50.0)	38.9 (35.4-42.8)	20.9 (18.0-23.0)	28.4 (25.0-74.8)	21.5 (16.7-29.6)
18:2	14.8 (8.1-22.5)	26.2 (16.0-35.0)	39.9 (33.7-41.1)	22.6 (20.2-24.0)	44.7 (33.3-52.3)	62.0 (52.5-65.8)
20:4	<1.0	<1.0	3.7 (2.1-4.8)	<1.0	<1.0	4.0 (3.6-4.5)
Phospholipids						
16:0	22.2 (16.9-28.2)	19.5 (16.0-22.1)	14.9 (12.7-17.7)	32.9 (27.6-37.4)	19.7 (16.9-23.4)	16.7 (12.8-21.2)
16:1	<1.0	<1.0	<1.0	5.6 (4.5-7.1)	<1.0	<1.0
18:0	21.9 (16.0-31.4)	22.4 (20.1-24.7)	26.0 (23.1-29.7)	23.7 (18.5-30.5)	28.6 (24.6-36.4)	26.8 (20.2-35.6)
18:1	33.2 (23.4-42.4)	31.2 (29.2-35.9)	29.6 (26.0-31.2)	14.5 (10.1-19.2)	18.1 (13.2-22.5)	17.8 (14.0-23.1)
18:2	18.5 (10.9-21.8)	21.3 (16.0-26.0)	19.3 (16.3-23.2)	15.4 (10.4-23.2)	27.5 (23.8-30.6)	28.7 (26.2-32.6)
20:4	<1.0	4.9 (3.9-8.0)	9.2 (8.7-11.1)	<1.0	7.5 (7.0-8.4)	9.9 (3.3-11.6)
Triglycerides						
16:0	25.1 (22.2-28.9)	24.5 (23.0-26.4)	24.6 (23.7-25.3)	30.7 (26.6-34.3)	21.9 (19.4-23.1)	22.3 (21.7-23.0)
16:1	3.8 (1.3-5.8)	3.1 (1.9-5.3)	6.6 (4.5-9.5)	8.8 (7.9-9.7)	4.0 (1.1-7.6)	7.6 (5.3-10.1)
18:0	37.7 (24.6-41.8)	45.9 (40.6-49.9)	31.1 (22.2-42.2)	21.9 (16.4-29.4)	38.4 (30.0-42.2)	19.4 (14.6-25.7)
18:1	25.5 (19.5-38.9)	22.2 (20.1-27.6)	22.3 (20.1-26.4)	25.4 (19.1-28.6)	23.5 (16.9-26.4)	21.1 (19.6-20.3)
18:2	2.0 (0.2-3.1)	4.4 (2.2-8.6)	7.9 (6.3-11.3)	9.1 (7.6-10.9)	9.7 (6.6-14.3)	23.7 (14.5-30.2)

[a]Mean values (weight percentages of total) and ranges for five animals are given in each instance. For identification of abbreviations, see Table II.

concentrations of the major plasma lipids at higher intakes of tallow and polyunsaturated fatty acid containing diets, increased concentrations of ruminant plasma lipids have been observed (21,39-41). As a result of feeding diet B in the present experiment, the concentration of triglycerides within the VLDL fraction was considerably increased and there was a compensatory decrease in the proportion of cholesteryl esters. With the addition of "protected" PUFA to the diet of fattening steers, a similar redistribution of lipids in favor of the low density fractions has also been observed (41). More recently it has been shown with rats that long term feeding of diets containing a high proportion of PUFA may have a specific effect upon the distribution of lipid classes and thereby the physical properties of the VLDL fraction (30). Thus, in common with the present observations with sheep, it was shown that by feeding diets high in safflower oil, the proportion of triglycerides specifically within the VLDL fraction was increased and there were concomittant changes in the distribution of the plasma lipoproteins in favor of the less dense fractions.

In the sheep which received diet B, the extensive increases which occured in the 18:2 concentrations within the lipoprotein fractions included a considerable increase in the 18:2 concentration within the triglycerides of the VLDL fraction. This was in contrast to the sheep which received diet D where the changes which occurred in the 18:2 concentrations within the plasma did not extend to the triglycerides of the VLDL. The differences in concentration and fatty acid composition of the VLDL triglycerides produced in the present experiment between the feeding of diets B and D are entirely consistent with the changes that have been extensively observed to occur in ruminant milk fat composition as a result of feeding such diets. Thus, in the ruminant animal, it is generally accepted (42.43) that the provision of long chain fatty acids for milk fat production occurs predominantly through the triglycerides contained within the lipoproteins of density less than 1.006. Furthermore, it has been shown that the changes in the 18:2 concentrations within the milk fat which occurred as a result of feeding a "protected" PUFA diet could be accounted for specifically by the changes produced in the concentration and 18:2 content elicited within the triglycerides of the VLDL and chylomicron classes (39). Conversely, the failure of an unprotected PUFA diet to produce any effect on milk fat composition has in turn been associated with an inability to influence, for any length of time, the 18:2 concentration of this plasma lipoprotein fraction.

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Dietary Control of the Chain Elongation of Palmityl-CoA in Rat Liver Microsomes

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ABSTRACT

The rate of chain elongation of palmityl-CoA to stearyl-CoA in rat liver microsomes was studied in connection with the nutritional status of the rats. The microsomal chain elongation activity, which had been decreased by starvation for 48 hr, was rapidly increased to a high level on refeeding. The apparent Km value for malonyl-CoA in both normal and refed rats was the same, 1.2×10^{-4}M. Both cycloheximide and actinomycin D prevented the induction of microsomal chain elongation activity which was associated with refeeding. In addition, the activity of acyl-CoA hydrolase and the rates of esterification of acyl-CoA into phospholipids and neutral lipids in microsomes were not changed by the dietary alteration. These results support the conclusion that changes of the activity of microsomal chain elongation of palmityl-CoA in various nutritional status result from a rapid synthesis of new enzyme(s).

INTRODUCTION

Palmityl-CoA is converted to stearyl-CoA by the chain elongation system in liver microsomes (1-3) and it has been established that alteration of dietary conditions caused considerable changes in the chain elongation activity (4-6). The response of acyl-CoA desaturation to dietary conditions has been studied by many laboratories. The desaturation activity decreased by starvation was rapidly restored to a high level on refeeding (7-9). This induction of the desaturase was assumed to be DNA-directed protein synthesis (9,10). However, little is known about how dietary alterations influence the rate of chain elongation of palmityl-CoA.

The experiments reported here were designed to determine whether the changes in the chain elongation activity, under different nutritional conditions, are linked to changes in protein synthesis.

MATERIALS AND METHODS

[1-^{14}C]Palmityl-CoA (58.2 Ci/mole), [1-^{14}C]stearyl-CoA (62.0 Ci/mole) and [2-^{14}C]malonyl-CoA (32.1 Ci/mole) were obtained from New England Nuclear Corp. (Boston, MA). Palmityl-CoA, stearyl-CoA, malonyl-CoA, actinomycin D and bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO), NADH and NADPH from Kyowa Hakko Co. (Tokyo, Japan), and cycloheximide from Wako Pure Chemical Industries (Tokyo, Japan).

Female albino rats of Wistar strain, weighing 80-130 g, were used. Rats were maintained on a commercial cube diet (Oriental Yeast Co., Tokyo, Japan) ad libitum. Starvation was induced by the deprivation of diet for 48 hr, and then refeeding was initiated with the commercial diet. When it pertained, cycloheximide dissolved in 0.9% NaCl or actinomycin D dissolved in 50% propylene glycol–0.9% NaCl was injected intraperitoneally at a dose of 0.2 mg (cycloheximide) or 0.15 mg (actinomycin D) per 100 g of body weight.

Preparation of Microsomes

At each experimental point, three or four rats were killed by decapitation. Livers were excised and pooled. The livers were homogenized in 3 volumes of cold 0.25 M sucrose solution. The homogenate was centrifuged at 20000 g for 20 min; the resulting supernatant was centrifuged at 105000 g for 60 min and the microsomal pellet was suspended in 0.25 M sucrose. An aliquot of the suspension was centrifuged at 105000 g for 60 min. The resulting pellet was resuspended in 0.25 M sucrose and used as microsomal preparation I. Another aliquot of the suspension was fractionated through a discontinuous sucrose gradients technique as described by Brophy and Gower (4). The final pellet was suspended in 0.25 M sucrose and was used as microsomal preparation II. Unless otherwise noted, preparation I was used throughout the experiments.

Thin Layer Chromatography

The chloroform extract of lipids was fractionated into individual components by thin layer chromatography (TLC) on silica gel. The developing solvent system was chloroform-methanol-water (65:25:4, v/v). The lipids were located by staining with iodine vapor. Identification was made by reference to standards run

simultaneously. The area corresponding to each lipid was scraped off and radioactivity was counted as described previously (14).

Enzyme Assays

The microsomal chain elongation of palmityl-CoA was assayed as described previously (6,7,14).

The fatty acyl-CoA hydrolase activity in liver microsomes was determined by a partially modified procedure of Jones et al. (15). The incubation mixture contained in a final volume of 0.5 ml: 20 nmoles of [1-^{14}C]palmityl-CoA (0.05 μCi) or [1-^{14}C]stearyl-CoA (0.05 μCi), 45 μmoles of Tris-HCl buffer (pH 7.4) and 0.5 mg of microsomal protein. Incubation was carried out at 30 C for 2 min. After the reaction was stopped by the addition of 0.2 ml of 4N H_2SO_4, the incubation medium was extracted three times with 3 ml of n-pentane. The pentane extracts were combined and evaporated to dryness with flushing of N_2. The free fatty acids obtained were dissolved in a constant volume of n-hexane. An aliquot of the hexane solution was used for the determination of radioactivity. The radioactivity was measured by a Packard Tri-Carb liquid scintillation spectrometer.

Incorporation of exogenously added acyl-CoA into lipids was assayed as follows. Incubation medium contained in a final volume of 0.5 ml: 22.5 nmoles of [1-^{14}C]palmityl-CoA (0.05 μCi) or [1-^{14}C]stearyl-CoA (0.05 μCi), 45 μmoles of Tris-HCl buffer (pH 7.4) and 0.5 mg of microsomal protein. Incubations were carried out at 37 C for 6 min. The reaction was stopped with chloroform-methanol (2:1, v/v) and lipids recovered in the chloroform phase were analyzed by TLC.

RESULTS AND DISCUSSION

The effect of dietary alterations on chain elongation activity of rat liver microsomes was recently reported by Sprecher (5) and our laboratory (6,7). Figure 1 shows the time course of the changes in microsomal chain elongation activity of palmityl-CoA when rats were refed after 48 hr starvation. The chain elongation activity was increased by refeeding until after 24 hr it was about five times that of starved controls.

In order to examine the nature of the chain elongation system, we studied the kinetics of the chain elongation of palmityl-CoA, using various concentrations of malonyl-CoA. As shown in Figure 2, the apparent Km values for malonyl-CoA of microsomes from normal and refed rat liver were approximately equal, and

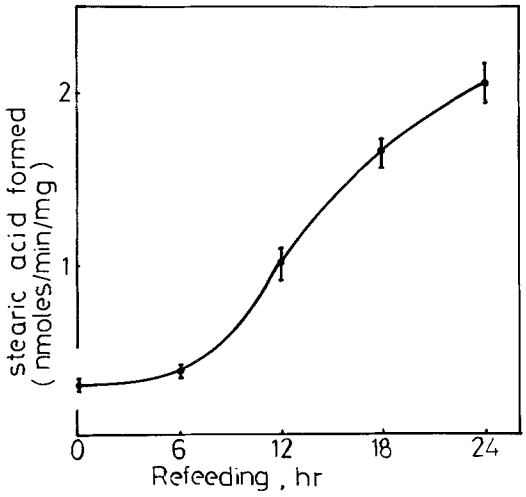

FIG. 1. Effect of refeeding on microsomal chain elongation activity of palmityl-CoA. The assay system contained in a final volume of 1.0 ml: 46.7 nmoles of [1-^{14}C]palmityl-CoA, 200 nmoles of malonyl-CoA, 3 μmoles of NADH, 3 μmoles of NADPH, 1 μmole of KCN, 90 μmoles of phosphate buffer (pH 7.4) and 1 mg of microsomal protein. Incubation was carried out at 37 C for 6 min under N_2. Each experimental point in the figure represents means of three separate experiments of three or four livers pooled each. Standard deviations are shown as vertical bars.

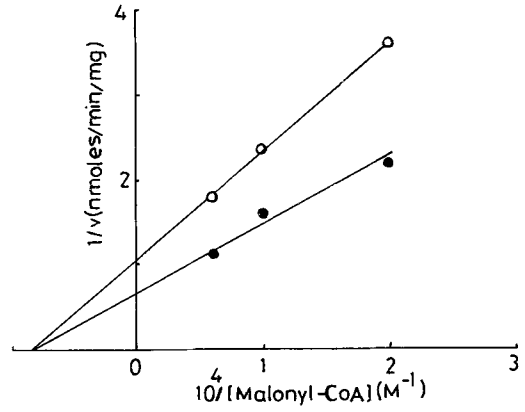

FIG. 2. Plot of reciprocal of reaction velocity (1/v) against (1/s) for chain elongation. •—•, microsomes from 24 hr refed rat liver; ○—○, microsomes from normal rat liver.

this value was about 1.2 x 10^{-4}M. This suggests that the enzyme for chain elongation in microsomes from the rat liver may be qualitatively the same in various nutritional conditions.

The rate of desaturation of fatty acyl-CoA, in response to various dietary conditions, has been studied by many laboratories (8,10) and it has been shown that changes of desaturation

TABLE I

Effect of Cycloheximide or Actinomycin D on the Induction of Microsomal Chain Elongation Activity by Refeeding[a]

Conditions	Chain elongation activity (Stearic acid formed, nmoles/min/mg of protein)
Untreated (Refed rat)	2.00 ± 0.26
Cycloheximide treated	0.73 ± 0.25
Actinomycin D treated	0.91 ± 0.09

[a]Cycloheximide (0.2 mg per 100 g body weight) or actinomycin D (0.15 mg per 100 g body weight) was injected intraperitoneally 12 hr after the initiation of refeeding, and animals were sacrificed 12 hr after the treatments (24 hr after the initiation of refeeding). Assay conditions for chain elongation were the same as in Figure 1. The activity represents a mean value ± SD of three of four separate experiments of three or four livers pooled each.

activity by dietary alterations were attributable to a DNA-directed protein synthesis. In the case of variability of chain elongation activity, however, all previous studies were accomplished without knowing the process of enzyme synthesis. Table I shows the effect of cycloheximide and actinomycin D on the induction of chain elongation activity. The drugs were injected intraperitoneally to the rats 12 hr after the initiation of refeeding. The same dosages of these drugs were sufficient to inhibit the induction of the activity of stearyl-CoA desaturase (10) and we also obtained the same results by these treatments (not shown). As can be seen in Table I, the cycloheximide treatment inhibited the induction of the activity of palmityl-CoA chain elongation. Actinomycin D treatment also inhibited the induction of chain elongation activity.

It is well known that rat liver microsomes possess acyl-CoA hydrolase (14-16), deacylation-reacylation systems for phospholipid biosynthesis (17), and esterification systems using mono- or diacylglycerol as acceptors (18) and that cholesterol is also esterified in the microsome. In addition, as we reported previously, palmityl-CoA chain elongation was inhibited by the end-product, stearyl-CoA (14). Therefore, if the activity of the enzymes mentioned above were affected by dietary alterations, the amount of palmityl-CoA available for chain elongation may affect the activity of microsomal chain elongation. At the same time, the amount of stearyl-CoA may feedback and inhibit chain elongation. To examine the fate of reaction product of chain elongation, we studied the distribution of stearyl-CoA, which was newly formed from palmityl-CoA and [2-^{14}C] malonyl-CoA by chain elongation, among various lipid classes. In this experiment, to eliminate the contamination of de novo fatty acid synthetase activity in cytosol, we used the microsomal preparation obtained by centrifugation through discontinuous sucrose density gradients. We also analyzed the species of fatty acids formed by using a radio-gas liquid chromatographic technique (6) and found that ca. 90% of the radioactivity was found in the stearic acid fraction. As shown in Table II, in all lipid classes the amount of newly formed stearic acid from starved rat liver microsomes was lower than that from refed rat liver microsomes. However, the distribution pattern of newly formed stearic acid among lipid classes

TABLE II

Distribution of Radioactivity in Lipid Classes after Incubation of Palmityl-CoA and [2-^{14}C] malonyl-CoA with Liver Microsomes from Starved or Refed Rats[a]

Microsomes	[2-^{14}C] Malonyl-CoA incorporated into lipids (nmoles)				
	Total	PC	PE	FFA	NL
Starved	1.74	0.43	0.60	0.45	0.14
	(100)	(24.9)	(34.3)	(26.1)	(8.1)
Refed	4.00	0.90	1.02	1.36	0.38
	(100)	(22.6)	(25.4)	(33.9)	(9.5)

[a]Incubation medium contained in a final volume of 0.5 ml: 22.5 nmoles of palmityl-CoA, 100 nmoles of [2-^{14}C] malonyl-CoA (0.1 μCi), 1.5 μmoles of NADH, 1.5 μmoles of NADPH, 0.5 μmoles of KCN, 45 μmoles of Tris-HCl buffer (pH 7.4) and 0.5 mg of microsomal protein (preparation II). Incubation was carried out at 37 C for 6 min under N_2. The reaction was stopped with 10 ml of chloroform-methanol (2:1, v/v) and lipids were extracted by the procedure of Folch et al. (19). The lipids recovered in chloroform phase was analyzed using thin layer chromatography as described in the text. The values in brackets represent the percentage of radioactivity recovered in chloroform phase. The values in the table were means of two separate experiments of three livers pooled each.

Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; FFA, free fatty acids; NL, neutral lipids other than free fatty acids.

TABLE III

Incorporation of Palmityl- and Stearyl-CoA into Various Lipids and Acyl-CoA Hydrolase Activity in Liver Microsomes from Starved and Refed Rats[a]

Acyl-CoA	Microsomes	Incorporation of acyl-CoA into lipids (nmoles) Total	PC	PE	FFA	NL	Acyl-CoA hydrolase (nmoles/min/mg)
Palmityl-CoA							
	Starved	15.8	0.82	0.79	13.73	0.61	7.04
		(100)	(5.2)	(4.0)	(86.9)	(3.9)	
	Refed	17.4	1.31	0.59	15.00	0.51	7.30
		(100)	(7.5)	(3.4)	(86.0)	(2.8)	
Stearyl-CoA							
	Starved	15.4	0.82	0.58	13.45	0.54	5.60
		(100)	(5.2)	(3.8)	(87.5)	(3.5)	
	Refed	15.2	1.18	0.69	12.82	0.47	5.39
		(100)	(7.8)	(4.6)	(84.5)	(3.1)	

[a]Assay conditions for incorporation of acyl-CoA into lipids and acyl-CoA hydrolase were described in the text. The values in brackets represent the percentage of radioactivity in chloroform phase. The values in the table were means of two separate experiments of three livers pooled each.

was the same as that of refed rats.

Table III shows the acyl-CoA hydrolase activity in liver microsomes from starved and refed rats. Both microsomes contained the same activities of acyl-CoA hydrolase for palmityl-CoA and stearyl-CoA. Furthermore, the rates of incorporation of both exogenously added palmityl-CoA and stearyl-CoA into phosphatidylcholine, phosphatidylethanolamine, and esterified neutral lipids were also unchanged by the dietary alteration. However, the distribution of stearic acid among various lipid classes differed considerably from the data described in Table II. Stearyl-CoA formed by chain elongation was incorporated more preferentially into phospholipids in comparison with stearyl-CoA added exogenously. These observations indicate that newly formed acyl-CoA is transacylated more effectively than that added exogenously to the incubation medium.

These results support the possibility that the changes in palmityl-CoA elongation activity are related only to the changes of enzyme(s) of the chain elongation system.

The data presented here are in agreement with earlier work (5-7) which shows that the fatty acid chain elongation system of rat liver microsomes is inducible by dietary alterations. It is suggested from the data in Table I that the marked increase in the activity of chain elongation of palmityl-CoA by refeeding was mediated by the DNA-directed new enzyme synthesis.

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The Phospholipids of Rabbit Type II Alveolar Epithelial Cells: Comparison with Lung Lavage, Lung Tissue, Alveolar Macrophages, and a Human Alveolar Tumor Cell Line

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ABSTRACT

The phospholipid composition of type II alveolar epithelial cells from the rabbit was compared with that of alveolar macrophages, lung lavage and lung tissue. In addition, the phospholipid composition of a human alveolar tumor cell line, which is morphologically similar to type II cells, was examined. Phosphatidylcholine accounted for 48% of the total phospholipid in the type II cells, 41% in the tumor cells, and 30% in the macrophages. Phosphatidylcholine was 51% disaturated in the type II cells, 54% in lung lavage, 39% in whole lung, 29% in lavaged lung and macrophages, and 16% in the tumor cells. Palmitic acid was the major fatty acid in phosphatidylcholine from all samples with the exception of the tumor cells in which almost half of the fatty acids were accounted for by oleic acid. The phospholipids of the type II cells were more similar to those of lung lavage, and thus surfactant, than to lung tissue and macrophages. This is consistent with their supposed role in surfactant production. The tumor cells, although morphologically similar to type II cells, were quite different with respect to phospholipid composition.

INTRODUCTION

There is considerable indirect evidence that the type II alveolar epithelial cell is the source of pulmonary surfactant, the phospholipid-rich material which lines the alveolar surface (1). Direct evidence has been lacking since isolated type II cells were not available. Recently, however, procedures for the isolation of type II cells have been developed in a number of laboratories (2-4). If type II cells are indeed the source of surfactant, it would be expected that they would contain relatively large amounts of phosphatidylcholine, particularly its disaturated species which is the major surface-active component of surfactant (1). The purpose of this investigation was, therefore, to determine the phospholipid composition of isolated type II cells and compare it with that of lung lavage, a rich source of surfactant, as well as with lung tissue and another readily available lung cell, the alveolar macrophage.

In addition, since a number of alveolar tumor cell lines with morphological characteristics of type II cells have been suggested as possible models for surfactant synthesis (5-8), isolated type II cells were compared with one such cell line with respect to phospholipid composition.

EXPERIMENTAL PROCEDURE

Adult rabbits (New Zealand White) were sacrificed with intravenous sodium pentobarbital. Type II alveolar epithelial cells and alveolar macrophages were prepared essentially by the method of Kikkawa et al. (9) except that in addition to the macrophages obtained by alveolar lavage, those removed prior to enzymatic digestion of the lung tissue were also collected. Combined macrophages were collected above a 1.07 Ficoll-Joklik density gradient (9). Purity of the cell populations was routinely monitored by examining air-dried smears subjected to Wright's stain (10) and was found to be 80-95% for type II cells and 65-80% for macrophages. Macrophages and, to a lesser extent, lymphocytes were the contaminants in the type II cell preparations while lymphocytes and some polymorphonuclear neutrophils contaminated the macrophage preparations. Type II cells and macrophages from 1-3 rabbits were combined for analysis. The alveolar tumor cells were a subclone of the A549 tumor cell line derived from a human lung carcinoma as described by Lieber et al. (5). They were cultured in Ham's F12K Medium (Grand Island Biological Company, Grand Island, NY) containing 10% fetal calf serum, penicillin (100 units/ml), streptomycin (100 μg/ml), and Fungizone (5 μg/ml). The cells were harvested when cultures were confluent. All cells were stored at -25 C prior to lipid extraction. Fresh lungs were immediately lavaged in situ via the trachea with five 50 ml aliquots of 0.9% NaCl. The lavage was centrifuged at 1000 x g for 15 min to remove cellular material and extracted with chloroform and

TABLE I

The Phospholipid Content and Composition of Type II Cells, Alveolar Macrophages, and Alveolar Tumor Cells[a]

	Type II cells	Alveolar macrophages	Tumor cells
Number of samples	4	5	3
Phospholipid content	(μg phospholipid phosphorus/mg protein)		
	8.4 ± 3.1	6.8 ± 1.1	7.6 ± 0.8
Phospholipid composition	(% of total phospholipid phosphorus)		
Phosphatidylcholine	47.5 ± 1.9	29.8 ± 0.7	41.3 ± 1.0
Phosphatidylethanolamine	10.9 ± 1.9	11.8 ± 0.5	28.0 ± 0.5
Sphingomyelin	9.8 ± 1.6	14.0 ± 0.4	6.3 ± 0.1
Phosphatidylinositol + phosphatidylserine	11.7 ± 2.0	12.2 ± 0.9	14.2 ± 0.6
Phosphatidylglycerol	4.3 ± 0.6	5.4 ± 0.4	2.0 ± 0.3
Cardiolipin	2.1 ± 0.4	4.8 ± 0.4	3.4 ± 0.2
Lyso-bis-phosphatidic acid	7.4 ± 1.4	12.9 ± 1.0	2.7 ± 0.5
Lysophosphatidylcholine	6.3 ± 1.5	9.1 ± 1.2	2.2 ± 0.2

[a]The data are the means ± SE from the number of samples indicated.

methanol by the procedure of Bligh and Dyer (11). The lavaged lungs, intact, nonlavaged lungs from additional animals, and the frozen cells were extracted with chloroform and methanol by the procedure of Folch et al. (12) as described previously (13).

The crude lipids were washed with 0.05 M NaCl (12). The phospholipid fraction, obtained by chromatography of the total lipids on a column of silicic acid (13), was fractionated into individual components by thin layer chromatography (TLC) on Quantum LQD plates (Quantum Industries, Fairfield, NJ) in chloroform-methanol-acetic acid-water (50:35:4:2 v/v), which resolved lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylinositol and phosphatidylserine combined, phosphatidylethanolamine, phosphatidylglycerol and cardiolipin combined, and lyso-bis-phosphatidic acid, and tetrahydrofuran-methylal-methanol-2M ammonia (10:8:2:1.1, v/v), which resolved phosphatidylethanolamine, cardiolipin, phosphatidylglycerol, and lyso-bis-phosphatidic acid from the remaining phospholipids. Phosphatidylcholine was isolated by TLC in chloroform-methanol-7 M ammonia (60:35:5 v/v) for subsequent fatty acid analysis and measurement of disaturated species. Phospholipids were eluted from the gel and quantitated by phosphorus assay (14) as described previously (13).

Disaturated phosphatidylcholine was separated from total phosphatidylcholine following osmium tetroxide treatment as described by Mason et al. (15). Phosphatidylcholine (1 mg) was applied in chloroform-methanol (20:1 v/v) to a 1.6 g column (1 cm ID) of neutral alumina (15). The column was washed with 10 ml of the same solvent and the disaturated species were eluted in 5 ml of chloroform-methanol-7 M ammonia (70:30:2 v/v) and quantitated by phosphorus assay (14). In this procedure, 96% of dipalmitoylglycerophosphocholine and 6% of dioleoylglycerophosphocholine was recovered in the disaturated fraction. In twelve experiments in which mixtures of dipalmitoylglycerophosphocholine and dioleoylglycerophosphocholine, ranging from 10% to 50% dipalmitoylglycerophosphocholine, were applied to the column, the recovery in the disaturated fraction was 113 ± 14% (SD). Thus, in this procedure the amount of disaturated phosphatidylcholine is somewhat overestimated. This appears to be due to elution of the oxidized phospholipid rather than unreacted unsaturated phospholipid since <1% of the fatty acids of the dioleoylglycerophosphocholine standard were saturated and 97% of the fatty acids of the recovered disaturated phosphatidylcholine from the lung materials were saturated.

Phosphatidylcholine was saponified and the fatty acids were methylated and analyzed by gas liquid chromatography as described previously (16). Fatty acid methyl esters were tentatively identified by comparing their relative retention times with those of standards.

Protein was measured by the procedure of Lowry et al. (17) using bovine serum albumin as standard.

Dipalmitoylglycerophosphocholine, phosphatidylethanolamine, and sphingomyelin were obtained from Sigma, St. Louis, MO. All other phospholipid standards were from Serdary, London, Ontario, Canada. Standard fatty acid methyl esters were from Supelco, Bellefonte, PA. Other chemicals were reagent grade or better.

TABLE II

Disaturated Phosphatidylcholine Content of Type II Cells, Alveolar Macrophages, Alveolar Tumor Cells, Lung Lavage, and Lung Tissue[a]

Type II cells	50.5 ± 2.1 (3)
Alveolar macrophages	29.3 ± 2.5 (5)
Tumor cells	16.0 ± 0.2 (2)
Lung lavage	54.0 ± 1.4 (5)
Intact lung	39.4 ± 1.8 (4)
Lavaged lung	28.6 ± 1.3 (5)

[a]The data are expressed as a percentage (± SE) of total phosphatidylcholine with the number of samples in parentheses.

RESULTS

The phospholipid content and composition of type II cells, macrophages and tumor cells is shown in Table I. The different cell types differed little in phospholipid content. There were differences in composition, however. Phosphatidylcholine accounted for ca. 50% of the total phospholipid in the type II cells, ca. 40% in the tumor cells, and only 30% in the macrophages. We have previously shown that phosphatidylcholine accounts for 80-90% of the total phospholipid in lung lavage; 73% in lamellar bodies, which are involved in the storage of surfactant prior to its release (18); and ca. 50% in lung tissue (13,16,18). The tumor cells contained almost three times as much phosphatidylethanolamine as the type II cells and macrophages while lyso-bis-phosphatidic acid was a relatively major component in the macrophages.

The amount of disaturated phosphatidylcholine in the three cell types is compared with that in lung lavage and lung tissue in Table II. About half of the total phosphatidylcholine in the type II cells and lung lavage was disaturated, 39% in the intact lung, 29% in the lavaged lung tissue and macrophages, and only 16% in the tumor cells.

The fatty acid composition of phosphatidylcholine is shown in Table III. Palmitic acid (16:0) was the major fatty acid in all samples examined with the exception of the tumor cells. It accounted for 63% of the total in lung lavage, 51% in lung tissue, 32% in the type II cells, and 19% in the macrophages. It accounted for 26% of the total fatty acids in the tumor cell phosphatidylcholine, but oleic acid (18:1), which accounted for 47% of the total, was the major component. Arachidonic acid (20:4) was not detectable in phosphatidylcholine from lung lavage, was present in small amount in that from type II and tumor cells, but accounted for 7% of the total fatty acids in phosphatidylcholine from the macrophages.

DISCUSSION

There are few reported studies on isolated type II cells. Kikkawa et al. (9) determined the phospholipid composition of type II cells from rabbit lung and compared it with that of macrophages. Their results are similar to those in this paper with respect to the relative amounts of total and disaturated phosphatidylcholine in both the type II cells and macrophages. Mason et al. (19) also reported a similar amount of total phosphatidylcholine in rabbit alveolar macrophages but less disaturated phosphatidyl-

TABLE III

Fatty Acid Composition of Phosphatidylcholine from Type II Cells, Alveolar Macrophages, Alveolar Tumor Cells, Lung Lavage, and Lavaged Lung[a]

Fatty acid methyl ester	Type II cells	Alveolar macrophages	Tumor cells	Lung lavage[b]	Lung tissue
Number of samples	4	3	3	3	3
14:0	2.9 ± 0.6	1.3 ± 0.9	0.1 ± 0.0	2.2 ± 0.1	1.7 ± 0.1
14:2	3.6 ± 0.5	3.4 ± 1.6	0.8 ± 0.1	N.D.[c]	2.6 ± 1.2
16:0	31.8 ± 1.8	19.2 ± 2.8	25.6 ± 0.5	62.8 ± 0.6	50.8 ± 0.9
16:1	12.0 ± 1.2	6.2 ± 2.2	11.4 ± 0.4	5.9 ± 0.8	4.5 ± 0.7
18:0	12.6 ± 0.6	11.1 ± 2.3	5.2 ± 0.3	2.5 ± 0.4	6.5 ± 0.4
18:1	10.5 ± 1.2	11.0 ± 1.7	47.4 ± 0.2	14.8 ± 0.6	14.4 ± 0.4
19:0	N.D.	10.1 ± 2.8	N.D.	N.D.	N.D.
18:2	19.0 ± 2.5	12.8 ± 0.9	1.9 ± 0.1	10.6 ± 1.0	17.3 ± 1.0
20:0	N.D.	3.7 ± 0.6	0.3 ± 0.1	N.D.	N.D.
18:3	3.2 ± 0.4	0.4 ± 0.4	2.2 ± 0.5	1.3 ± 0.1	N.D.
20:4	0.4 ±0.3	7.3 ± 0.5	0.4 ± 0.1	N.D.	2.2 ± 1.1
Unidentified	4.2 ± 2.6	13.5 ± 3.7	3.6 ± 0.2	N.D.	N.D.

[a]The data are expressed as a percentage (± SE) of the total fatty acids.

[b]The lung lavage data are from a previous study (16).

[c]N.D., not detectable.

choline–14% of the total compared with almost 30% in the present study and in that of Kikkawa et al. (9). The phospholipid composition of macrophages in the present study is otherwise similar to that reported by Mason et al. (19) with the exception that in the present study there is more lysophosphatidylcholine and less phosphatidylethanolamine. Some of these differences may be due to measurement of different macrophage pools. In the previous studies (9,19), for instance, only macrophages obtained from alveoli by a lavage procedure were used while in the present study interstitial macrophages were also included.

Since dipalmitoylglycerophosphocholine is the major distinguishing component of pulmonary surfactant (1), it is of interest to compare the amount of total and disaturated phosphatidylcholine and its content of palmitic acid in materials rich in surfactant with that in cells thought to be involved in surfactant synthesis (type II cells and possibly tumor cells) and cells not involved in surfactant production (alveolar macrophages). In lung lavage and lamellar bodies, materials rich in surfactant, phosphatidylcholine accounts for over 70% of the total phospholipid (16). In the type II and tumor cells it is only 40-50%, which is about the level in whole lung (13) and in a number of other organs (20). On the other hand, the type II cell is relatively rich in disaturated phosphatidylcholine. About half of its total phosphatidylcholine is disaturated. This is about the same percentage as in lung lavage. In whole lung, in contrast, only 39% of the total is disaturated. Similar values have been reported for whole lung from the rabbit (21) and the rat (15) by Mason et al.

The tumor cells, in contrast to the type II cells, contain very little disaturated phosphatidylcholine. This is also reflected in fatty acid composition. In phosphatidylcholine from the tumor cells oleic acid is the major fatty acid while in that from the other samples examined, palmitic acid is the major component. Previous studies on the A549 tumor cell line (5) indicated that about half of its phosphatidylcholine was disaturated. Whether this discrepancy is due to genetic or culture condition factors is not known. It will be of interest to compare type II and tumor cells cultured under the same conditions.

It is clear from the fatty acid composition that dipalmitoylglycerophosphocholine is not the only disaturated species of phosphatidylcholine in the type II cells. It is surprising that phosphatidylcholine from the type II cells contains even less palmitic acid than that from the lavaged lung tissue. However, it is known from previous studies (16) that the bulk of the palmitic acid-containing species of phosphatidylcholine are in the lamellar bodies rather than in other subcellular fractions of whole lung. Further studies are needed to elucidate the subcellular localization of individual molecular species of phosphatidylcholine in the type II cells.

These studies, therefore, show that the type II cells are rich in disaturated phosphatidylcholine. In this respect, they are similar to lung lavage and less like lung tissue and alveolar macrophages which is in keeping with their suggested role in surfactant synthesis. The tumor cells examined in this study, although morphologically similar to type II cells (5), are quite different with respect to phospholipid composition.

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SHORT COMMUNICATIONS

Hepatic Triglyceride Lipase Deficiency in Liver Disease

ABSTRACT

The activity of post-heparin lipases in patients with alcoholic hepatitis and viral hepatitis was evaluated. Lipoprotein lipase and hepatic triglyceride lipase were differentiated by assay under high and low salt conditions and also by separation on heparin-agarose affinity chromatography columns. The mean activity of hepatic triglyceride lipase in the sera of liver disease patients was only 21-24% of the mean of controls, but lipoprotein lipase in patients' sera was not different from normal levels. Hepatic triglyceride lipase deficiency may partially account for the accumulation of a triglyceride-rich low density lipoprotein in liver disease.

INTRODUCTION

It has been established that the lipolytic activity of post-heparin plasma (PHLA) is due to the presence of three component lipases (1). Lipoprotein lipase (LL) released from extrahepatic sites (primarily adipose tissue) is inhibited by high concentrations of either NaCl or protamine sulfate (2) and can be divided into two types on the basis of apoprotein activator requirements for either apo C-I or apo C-II (1). Another lipase purified recently (3) from post-heparin plasma has been localized in hepatic tissue (4,5). The hepatic triglyceride lipase (H-TGL) does not require activation by apoproteins, is resistant to protamine sulfate, and is stimulated by 1 M NaCl.

Lipoprotein lipase is thought to function in the hydrolysis of triglyceride-rich chylomicrons and very low density lipoprotein (VLDL) (6,7); however, a specific physiological role for H-TGL has not been identified. The localization of H-TGL in the liver, the site of lipoprotein remnant recycling (8), and its substrate specificities (9) suggest a possible role for H-TGL in the degradation of the "remnant" lipoproteins produced by the catabolism of VLDL and chylomicrons (10).

In a recent study, we have isolated a triglyceride-rich, cholesteryl ester deficient low density lipoprotein from the plasma of subjects with alcohol-induced liver injury (11). The cholesteryl ester deficiency was secondary to a decrease in lecithin:cholesterol acyltransferase (LCAT) activity, an enzyme synthesized in the liver. The accumulation in plasma of a lipoprotein similar in triglyceride composition to remnant lipoproteins suggested the possibility of impaired lipoprotein lipolysis due to decreased H-TGL and/or LL.

MATERIALS AND METHODS

Ten subjects (four male, six female) with liver disease were chosen from a general patient population. Each was judged to have either acute alcoholic liver disease or viral hepatitis on the basis of clinical evaluation and characteristic abnormal liver function tests. Nine normal subjects (five male, four female) served as controls and were roughly age and sex matched with the patients.

Following a 12 hr fast, 10 IU of sodium heparin (Riker Laboratories, Northridge, CA) per kilogram of body weight was injected intravenously as a bolus, and 10 min later a post-heparin sample was obtained from a different venipuncture site, placed into a heparinized tube, immediately centrifuged at 4 C to separate plasma, and stored at -70 C until assayed.

H-TGL was separated from LL by affinity

TABLE I

Hepatic Triglyceride Lipase Deficiency in Acute Liver Disease as Demonstrated by Heparin-Agarose Affinity Chromatography

	H-TGL (Peak I) (μmoles/ml/hr)	LL (Peak II) (μmoles/ml/hr)
Control (5)[a] $\bar{x}$ (range)	3.28 (1.55-5.59)	1.45 (0.29-2.47)
Liver disease (5) $\bar{x}$ (range)	0.70 (0.13-1.05)	1.47 (0.55-2.77)
$\frac{\text{Liver disease}}{\text{Control}} \times 100$	21.3	101.4

[a]Each value in the table represents the mean of five samples and the range of values found.

TABLE II

Direct Measurement of Plasma Post-Heparin Lipolytic Activity in the Presence of either High Salt ($PHLA^{HS}$) or Low Salt ($PHLA^{LS}$) Substrate

	$PHLA^{HS}$ [a] (μmoles/ml/hr)			$PHLA^{LS}$ [b] (μmoles/ml/hr)		
	Substrate preparation[c]			Substrate preparation		
	1	2	3	4	5	6
Control (9)[d] $\bar{x} \pm 1$ SEM	31.3 ± 4.0	13.3 ± 1.8	24.7 ± 3.1	13.2 ± 2.0	10.4 ± 1.6	13.7 ± 2.0
Liver disease (10) $\bar{x} \pm 1$ SEM	7.2 ± 1.4	3.0 ± 0.5	6.5 ± 1.2	8.1 ± 2.0	7.2 ± 2.0	8.9 ± 2.5
$\frac{\text{Liver disease}}{\text{Control}} \times 100$	23.0	22.7	26.3	61.4	69.2	65.0

[a]Under this condition lipoprotein lipase is completely inhibited and hepatic lipase is stimulated.

[b]This condition measures total lipoprotein lipase but is suboptimal for hepatic lipase.

[c]Substrate preparations 1 and 4, 2 and 5, 3 and 6 were prepared at 1 wk intervals and used to assay all control and liver disease samples on each date.

[d]Number of subjects in each group.

chromatography on a column (0.9 x 10 cm) filled to a depth of 1 cm with heparin-Sepharose prepared from CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals, Piscataway, NJ) (12). Plasma diluted with an equal volume of 0.45M NaCl in 0.005M barbital buffer was applied to the column and the lipases were eluted separately; H-TGL (Peak I) with buffered 0.72M NaCl and LL (Peak II) with buffered 1.5M NaCl (1). All buffers were at pH 7.0.

For lipase determination 0.1 ml whole plasma or column eluate was incubated at 28 C with 0.9 ml of high salt (HS) substrate for H-TGL or low salt (LS) substrate for LL. Substrates were prepared by a 5 min sonication of 70 mg (8.75 μC) triolein-1-14C (Applied Science, State College, PA) with 35 ml of either HS or LS buffer. Buffers were 0.2M Tris, 0.5% gum arabic, 0.5% bovine serum albumin, and either 0.75M NaCl (HS) or 0.075M NaCl (LS). Low salt buffer for assay of LL also contained 2.0 ml of pre-heparin plasma from controls as a source of activator apoprotein. After hexane extraction, radioactive free fatty acids were partitioned into 0.1M KOH (13) and counted by liquid scintillation spectrometry.

RESULTS AND DISCUSSION

A wide range of activity of H-TGL (Peak I) separated from plasma by heparin-Sepharose affinity chromatography was obtained in the five control samples studied (Table I). The range of H-TGL activity in liver disease patients was also large but there was no overlap between the control values and those obtained in the patients. The mean value of H-TGL in liver disease patients was only 21.3% of the mean value of the controls. In contrast, the mean LL (Peak II) activity of controls and patients was essentially the same and the ranges in each group showed wide overlap. Thus, by affinity chromatographic separation of H-TGL and LL, it appears that H-TGL is greatly decreased in liver disease but LL is essentially normal.

This conclusion was confirmed by the results of the direct measurement of plasma PHLA under conditions of high salt ($PHLA^{HS}$) and low salt concentrations ($PHLS^{LS}$). Using three different preparations of high salt substrate, in which LL is completely inhibited, but H-TGL stimulated, liver disease patients were found to have a mean H-TGL ($PHLA^{HS}$) of only 24% of controls (Table II). Different preparations of substrate showed large variations in absolute activity, particularly in the direct assay of plasma with high salt substrate. This was probably due to the difficulty in reproducing the sonication procedure. To circumvent this problem, we assayed all controls and samples with three different substrate preparations on separate occasions and each time the relative activities of controls and patients were the same. Correlation coefficients between activities of the group of plasma samples assayed with different substrate preparations were 0.97 or greater. Direct assay of PHLA in liver disease patients using low salt substrate gave a mean value for three substrate preparations of 65% of controls. This value is compatible with the assumption that LL is unchanged in liver disease and that the $PHLA^{LS}$ assay of liver disease plasma includes all LL activity but only 22-24% of H-TGL activity.

The decreased H-TGL in liver disease does not appear to be related to sex difference. The mean $PHLA^{HS}$ (all assays) for five male controls was 24.2 (μmoles/ml/hr) as compared to

21.7 for four female controls. Four male liver disease patients had a mean value of 6.0 compared to a mean of 5.3 for six females. There also was no difference between patients with viral hepatitis and those with alcoholic hepatitis. Five viral hepatitis patients had a mean $PHLA^{HS}$ of 23% of controls compared to 25% for five alcoholic hepatitis patients.

The present results raise some interesting speculations regarding a possible role for H-TGL in plasma lipoprotein metabolism. Hypertriglyceridemia in acute hepatic injury may be due primarily to an accumulation of a triglyceride-rich low density lipoprotein (LDL) (11). It would appear that this abnormal LDL is an intermediate of VLDL catabolism since its apoprotein content is similar to normal LDL (14) which is believed to be a product of VLDL catabolism. Under normal circumstances the triglycerides of VLDL are hydrolyzed, in part, by LL yielding a triglyceride-rich intermediate particle (7). At the same time, LCAT utilizes HDL as substrate to form cholesteryl esters which are incorporated by nonenzymatic exchange into the VLDL to LDL pathway. H-TGL may fit into this catabolic scheme by hydrolyzing the triglyceride-rich remnant particles as they circulate through the liver. In liver disease, a combination of decreased LCAT and H-TGL could result in accumulation of a triglyceride-rich, cholesteryl ester-deficient LDL. An abnormal low density lipoprotein of this composition had been found in subjects with liver disease who have low LCAT activity (11) and a lipoprotein of similar composition was isolated by Müller et al. (15) and Sauar et al. (16) from patients with liver injury. Thus, acute liver disease and the recovery period which follows provides a model system with which to study the relative role played by H-TGL, LL, and LCAT in lipoprotein catabolism.

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Stimulation of Hepatic Lipogenesis by Eicosa-5,8,11,14-Tetraynoic Acid in Mice Fed a High Linoleate Diet

ABSTRACT

Liver slices, from mice fasted for one day and then refed for three days either a 15% corn oil diet or a 15% corn oil diet containing eicosa-5,8,11,14-tetraynoic acid (TYA), were incubated with [1-^{14}C]acetate or [^{3}H]H_2O to determine lipogenic capacity. Dietary TYA produced a twofold stimulation in fatty acid and cholesterol synthesis. TYA also caused an increase in the relative proportion of linoleate ($C_{18:2}$) and a decrease in that of arachidonate ($C_{20:4}$) in liver. Thus, (a) despite high levels of $C_{18:2}$, hepatic lipogenesis can be increased, and (b) even short term feeding of TYA can alter the hepatic fatty acid composition presumably by inhibition of arachidonate synthesis from linoleate.

INTRODUCTION

The ability of dietary fat to control hepatic lipogenesis in adult animals has been known for many years (1-3) and has been studied by many investigators (4-8). Although all dietary fats, those which contain saturated as well as unsaturated fatty acids, are active in decreasing fatty acid synthesis (6,9,10), it is the naturally occurring polyunsaturated fatty acids which are most effective (9). Changes in the activities of enzymes associated with fatty acid synthesis are coincident with shifts in the fatty acid composition of liver. For example, when a fat-free or a linoleate deficient diet is fed, the linoleate level falls and a dramatic increase in the enzyme activities for fatty acid synthesis occurs in the liver (4,9). Upon feeding a linoleate containing diet, hepatic fatty acid synthesis is depressed and the levels of both linoleate and arachidonate are increased (4,9,11). Thus, it has not been possible to determine the effect on hepatic lipogenesis of high linoleate levels under conditions where arachidonate levels are low.

Recently we were able to produce a relatively high level of linoleate and a low level of arachidonate in the livers of mice by feeding a corn oil diet containing eicosa-5,8,11,14-tetraynoic acid (TYA) for 6 wk (12). Indeed, Coniglio et al. (13) have suggested that dietary TYA may be effective in inhibiting the conversion of linoleate to arachidonate by rat liver. In the present communication, we show that such a polyunsaturated fatty acid pattern can also be obtained by feeding the corn oil-TYA diet for even short periods of time. In addition, our results demonstrate that hepatic fatty acid synthesis can be stimulated despite the increased levels of linoleate if the levels of arachidonate are low.

EXPERIMENTAL PROCEDURES

C_3H female mice (20 g) were fasted for 1 day and were then fed for 3 days a fat-free diet, a 15% corn oil diet or those diets to which 0.033% TYA was added. The composition of these high carbohydrate diets was given previously (11).

TABLE I

Alteration of the Fatty Acid Composition of Livers by Feeding Eicosa 5,8,11,14-Tetraynoic Acid[a]

Fatty acid chain length	Diet: Fat-free, Alone (8)	Diet: Fat-free, + 0.033% TYA (4)	Diet: 15% Corn oil, Alone (8)	Diet: 15% Corn oil, + 0.033% TYA (5)
C_{14}	0.5 ± 0.1	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.04
C_{16}	24.9 ± 2.5	23.1 ± 2.8	23.6 ± 0.7	16.6 ± 1.4
$C_{16:1}$	7.4 ± 0.7	6.3 ± 0.8	2.9 ± 0.4	3.4 ± 0.5
C_{18}	7.1 ± 0.6	2.7 ± 0.9	8.3 ± 1.1	5.5 ± 1.0
$C_{18:1}$	48.7 ± 3.0	56.3 ± 3.4	29.0 ± 3.5	38.3 ± 2.7
$C_{18:2}$	4.0 ± 0.7	5.8 ± 1.4	21.9 ± 1.4	30.1 ± 1.2
$C_{18:3}$	1.3 ± 0.3	2.4 ± 1.0	0.9 ± 0.1	1.8 ± 0.3
$C_{20:3}$	2.3 ± 0.4	0	0	0.7 ± 0.2
$C_{20:4}$	3.9 ± 0.3	3.1 ± 0.4	13.1 ± 1.9	3.4 ± 0.4

[a]The results are averages and their standard errors of separate determinations with livers from the number of mice given in parentheses for each group.

Mice were killed by cervical fracture and their livers were quickly removed and chilled in ice-cold Krebs-Henseleit bicarbonate buffer (pH 7.4) (14). Slices of liver (100 mg, 0.4 mm thick) were incubated for 3 hr at 37 C in 2 ml Krebs-Henseleit bicarbonate buffer in the presence of [1^{14}C]acetate or [^{3}H]H_2O that served as substrates for lipid synthesis. The reaction was stopped by the addition of 1 ml 10% perchloric acid. Where appropriate, $^{14}CO_2$ was absorbed and assayed for radioactivity as given previously (15). The washed tissue was refluxed overnight with 1 ml 30% KOH in 50% methanol. Following acidification, the fatty acids and the sterols were extracted into hexane. An aliquot of the hexane extract was evaporated to dryness under a stream of nitrogen, and the digitonin-precipitable sterols were prepared and washed according to the method of Sperry and Webb (16). The term "cholesterol" refers to the digitonide precipitate. Radioactivity in fatty acids was determined by subtracting that recovered in cholesterol from that recovered in the hexane extract (15).

Another portion of liver (100 mg) was refluxed with 1 ml 30% KOH in 50% methanol. Fatty acids were extracted and converted to methyl esters with diazomethane (17). The types and percent composition of fatty acids were determined by gas liquid chromatographic procedures published previously (18).

RESULTS AND DISCUSSION

Previously, we reported (12) that when mice were fed a corn oil-TYA diet for 6 wk, their livers contained a relatively higher level of linoleate and a lower level of arachidonic acid than when TYA was omitted from the diet. The results in the present study reveal that such long term feeding of TYA is not necessary to alter the relative levels of these two polyunsaturated fatty acids in liver (Table I).

It is well known that when mice are fed a high carbohydrate, fat-free diet, the rate of hepatic fatty acid synthesis is several-fold greater than when a corn oil diet is fed. Feeding TYA with the fat-free diet caused no appreciable change in the lipogenic capacity of mouse liver (Table II). However, the inclusion of this compound in the corn oil diet resulted in a twofold increase (Table II). A comparison of the fatty acid composition of the livers of mice fed corn oil diets in the absence or presence of TYA (Table I) and their lipogenic capacity (Table II), indicates that an increased level of linoleate is not associated with reduced lipogenesis.

TABLE II

Effect of Dietary Eicosa-5,8,11,14-Tetraynoic Acid (TYA) on Hepatic Fatty Acid and Cholesterol[a]

Diet	Metabolic product from:							
	[1-^{14}C]acetate			[^{3}H]H_2O		[^{3}H]H_2O + acetate		
	CO_2	Fatty acids	Cholesterol	Fatty acids	Cholesterol	Fatty acids	Cholesterol
Fat-free	450 ± 55	226 ± 29	5.9 ± 0.6	1940 ± 343	50.5 ± 9.5	2058 ± 244	48.1 ± 10.7
Fat-free + 0.033% TYA	481 ± 68	217 ± 22	5.0 ± 1.1	2009 ± 281	42.4 ± 11.3	1946 ± 361	50.5 ± 16.5
15% Corn oil	310 ± 34	76 ± 10	3.5 ± 0.6	618 ± 104	24.2 ± 2.8	660 ± 104	19.6 ± 1.8
15% Corn oil + 0.033% TYA	434 ± 57	149 ± 14	10.1 ± 2.5	1418 ± 267	62.2 ± 7.4	1359 ± 186	60.6 ± 9.7

[a]Liver slices (100 mg) were incubated with mechanical agitation for 3 hr at 37 C in 2 ml of Krebs-Henseleit bicarbonate buffer (pH 7.4) containing either 10 μmoles [1-^{14}C]acetate (0.5 μCi), [$^{3}H_2O$] (1 mCi), or 10 μmoles acetate and [^{3}H]H_2O (1 mCi) as indicated above. The gas phase was a mixture of 95% O_2 and 5% CO_2. All other experimental details are given in the text. Results are presented as nmoles of substrate converted to product per 100 mg of liver slices per 3 hr and represent the average values of five to seven experiments with different animals ± their standard errors.

TABLE III

Effect of Dietary TYA and Arachidonate on Hepatic Fatty Acid and Cholesterol Synthesis[a]

Diet	Metabolic products from: [1-^{14}C]acetate CO_2	[1-^{14}C]acetate Fatty acids	[1-^{14}C]acetate Cholesterol	[^{3}H]H_2O + acetate Fatty acids	[^{3}H]H_2O + acetate Cholesterol
Fat-free	924 ± 105	663 ± 87	6.9 ± 2.0	3538 ± 607	44.1 ± 8.0
15% Corn oil	895 ± 104	213 ± 29	5.0 ± 1.5	1275 ± 292	22.9 ± 4.9
15% Corn oil + 0.033% TYA	1007 ± 72	527 ± 40	5.6 ± 0.5	3059 ± 530	32.8 ± 4.2
15% Corn oil + 0.033% TYA + 0.25% Arachidonic acid	887 ± 73	357 ± 112	4.7 ± 1.3	1684 ± 269	23.3 ± 5.5

[a]Mice were fed the indicated diets for 4 wk prior to sacrifice. See Table II and text for additional experimental details. Values (nmoles of substrate converted to product per 100 mg of liver slices for 3 hr) recorded above are the average values for five to eight livers ± their standard errors.

As expected, the dietary treatments did not affect acetate oxidation to carbon dioxide; however, cholesterol synthesis, which was depressed by corn oil feeding, was restored to a level found in the livers of the fat-free control group by the inclusion of TYA in the diet (Table II). Thus, dietary TYA appears to reverse the decrease in both fatty acid and cholesterol synthesis in the livers of mice fed diets high in corn oil. Coincident with the increased hepatic lipogenesis in mice fed the corn oil-TYA diet is the decrease (50%) in the arachidonic levels of the liver (Table I). The relative proportions of arachidonate in the livers of mice fed the corn oil-TYA diet are similar to those in the livers of mice fed a fat-free diet. Thus, it would appear that an increased lipogenic capacity is associated with a decreased arachidonate level.

Having satisfied ourselves that TYA effectively reduces the amount of arachidonate in the liver while increasing its linoleate content, we studied hepatic lipogenesis in mice which were fed for 4 wk either the corn oil-TYA diet or the same diet with 0.25% arachidonate. Fatty acid synthesis from both [1-^{14}C]acetate and [^{3}H]H_2O by liver slices prepared from mice fed the latter diet was reduced by 30-40% (Table III). Indeed, when compared to the fat-free carbohydrate diet, the corn oil-TYA-0.25% arachidonate diet produced a twofold decrease in hepatic fatty acid synthesis (Table III).

Although dietary TYA can substantially relieve the inhibition of hepatic lipogenesis caused by dietary linoleate, it does not restore this activity fully to the levels observed after feeding a fat-free diet. This would indicate that in addition to the levels of arachidonic acid, other factors such as those which cause depressed lipogenesis after feeding a saturated fat diet must also play a role in the regulation of hepatic lipogenesis.

Although a great many investigations on lipogenesis have been carried out, our understanding of the specific mechanism by which this process is controlled is not yet clearly understood. In 1963, we demonstrated that in as little as 4 hr after corn oil administration, hepatic acetyl-CoA carboxylase, a rate limiting enzyme in fatty acid synthesis, was inhibited (5). We suggested at that time that products of dietary triglycerides might act as effective inhibitors of lipogenesis within the cell. Indeed, subsequently, other workers have shown that both free fatty acids (19) and long chain acyl-CoA derivatives (20,21) can inhibit the activity of acetyl-CoA carboxylase. The present studies suggest that arachidonate or its metabolic products are potent inhibitors of the synthesis and/or activities of the enzyme(s) involved in lipogenesis.

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Phosphono-Lipid Content of the Oyster, *Crassostrea virginica*, in Three Physiological Conditions

ABSTRACT

The distribution of lipid-bound phosphorus between the phosphodiester and phosphonate forms was determined for the oyster in three physiological conditions. The adductor muscle had the highest level of phosphonate phosphorus, 92 μg/g wet weight tissue, or 40.4% of all muscle phospholipids. The total phospholipid level (μg/g wet weight tissue) increased in all tissues of the oyster during starvation, as did the percentage of phosphonolipids in all tissues except the adductor muscle, in which it remained constant. These data suggest that during starvation the phosphonate bonds are conserved at the expense of phosphodiester bonds in oyster lipids.

INTRODUCTION

The discovery of naturally occurring phosphonates (1) led to investigations of their biological distribution and metabolism. Studies of their phylogenetic distribution have indicated that phosphonates are found generally throughout the animal phyla, and that certain marine organisms contain high concentrations of these compounds (2,3). Biosynthetic (4) and degradative (5) reactions involving this bond, as well as incorporation of ^{32}P labeled aminoethylphosphonate into phospholipids, have been described (6,7).

Despite the interest in lipid-bound phosphonates, the physiological role of these metabolites has remained unclear. An assessment of the biological function of phosphonates may be begun by monitoring the change in phosphonate content of an organism as its physiological condition is varied.

Among the animals known to contain phosphonates, few have a higher lipid-bound phosphonate content than the American oyster *Crassostrea virginica*, with 25-35% of its phospholipid as phosphonate (3,8). This observation and the ease of maintaining the oyster in the laboratory (9) prompted its selection for the present study in which the gross distribution of lipid-bound phosphorus, as ester phosphorus and phosphonate, was measured in oysters in three distinct conditions.

MATERIALS AND METHODS

Oysters (3-5 cm in height and 3-4 cm long) were obtained from a commercial source (Chesapeake Oyster Culture Co., Shadyside, MD) in late March and late July. Oysters obtained in March were divided into two groups. One group (starved) of about fifty oysters was held in the laboratory in 12 parts per thousand artificial sea water ("Instant

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TABLE I

Distribution of Lipids Phosphorus in Oysters in Three Physiological Conditions

Tissue	Condition	Total lipid phosphorus	Ester phosphorus	Phosphonate phosphorus	Percent phosphorus	
		(μg P/g wet weight tissue)			Ester phosphorus	Phosphonate
Total oyster	Fresh	237	182	55	76.8	23.2
	Starved	313	207	106	66.1	33.9
	Post spawning	212	135	77	63.7	36.3
Mantle and gills	Fresh	267	203	64	76.0	24.0
	Starved	281	235	146	61.7	38.3
	Post spawning	209	149	60	71.3	28.7
Adductor muscle	Fresh	228	136	92	59.6	40.4
	Starved	289	177	112	61.2	38.8
	Post spawning	156	115	41	73.7	26.3
Digestive diverticula	Fresh	242	211	31	87.2	12.8
	Starved	404	294	110	72.8	27.2
	Post spawning	297	211	86	71.0	29.0

Ocean," Aquarium Systems, Inc., Eastlake, OH) at 18 C (9) for 37 days. During this time no attempt was made to feed the animals; the saline was changed daily. The rest of the March oysters (fresh) and all of the July oysters (post spawning) were sacrificed immediately.

The three groups were treated as follows. Oyster tissue, either whole oysters or animals separated into mantle and gills, adductor muscle, and digestive diverticula, was drained on filter paper for several minutes. All tissues were extracted according to Folch et al. (10) with the aid of a Sorvall Omni-Mixer (Dupont Instruments, Newtown, CT). Upon completion of the Folch wash, the crude lipid extracts were reduced in volume and stored in chloroform-methanol 2:1 (v/v) at -10 C until analyzed.

Ester phosphorus was estimated by the method of Bartlett (11) holding the addition of hydrogen peroxide to a minimum; total phosphorus was estimated by the Aalbers and Bieber procedure (12). Phosphonate content of extracts is the computed difference between total phosphorus and ester phosphorus values.

RESULTS AND DISCUSSION

The distribution of lipid-bound phosphorus between the phosphodiester and phosphonate forms in the various oyster tissue is shown in Table I. The phosphono-lipid content of whole oysters varied between 23% and 36% of total lipid phosphorus, supporting previously published data (3,8). In addition, it may be seen that the American oyster is similar to the European oyster, in that the adductor muscles of both species contain about 40% phosphonate phosphorus (13).

The oysters used in this study were in three distinct physiological conditions. Oysters harvested in March were at the annual maximum content of both glycogen and protein (14). After 37 days in the laboratory with no food, these oysters were in a starved condition, having mobilized their glycogen stores. Oysters harvested in July were just at the end of their spawning cycle and at the annual minimum in glycogen (14).

Comparison of the total lipid phosphorus values of oysters in these conditions reveals that starved oysters contain more phospholipid per gram wet weight than do fresh or spawning oysters. This may be partially explained by noting that higher glycogen contents will increase tissue weight thereby decreasing content of other components measured on a weight basis. Starved oysters would have lower tissue weight due to decreased glycogen content, thus explaining the apparent increase in total lipid phosphorus per gram wet weight of starved oysters.

The content of phosphono-lipids showed an increase in the starved oysters, as reflected in both the quantity of phosphorus and the percentage of total lipid-bound phosphorus (Table I). The percentage of ester lipid phosphorus decreased in the starved and post spawning groups. As the oysters deplete the glycogen depots, the phosphono-lipids are conserved at the expense of phosphodiester phospholipids.

It has been suggested that phosphono-lipids confer some biological advantages on the organisms that possess them. Assuming that the phosphone-lipid contributes to some important property of membrane function (2), the relative resistance of these lipids to catabolic processes assures that this function will be maintained

even during periods of stress, such as starvation. In other words, even under conditions of deprivation of exogenous carbon and phosphorus sources, the phosphono-lipids will not be used as such a source and will maintain their constitutive function.

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[Received January 20, 1977]

Bis-Phosphatidic Acid in Developing Soybeans and Soybean Suspension Cultures

ABSTRACT

A phospholipid which rapidly accumulates radioactivity from [1-^{14}C]acetate administered to slices of developing soybeans or to suspension cultures of soybean cells was isolated. Its structure was identified by comparison of its properties and degradation products with those of authentic lipid standards using infrared absorption spectrometry, thin layer chromatography, acetolysis, mild alkaline hydrolysis, and determination of molar ratios of phosphorus, glycerol, and acyl moieties. The structure of the phospholipid was found to be *bis*-phosphatidic acid, a phospholipid previously unreported in higher plants.

INTRODUCTION

We previously isolated an unidentified phospholipid of high specific activity from slices of developing soybeans (1) and from soybean suspension cultures (2) which had been incubated with [1-^{14}C]acetate. Others reporting similar observations on incubation of soybean tissues with radioactive precursors have implicated N-acyl phosphatidylethanolamine (NAPE) on the basis of chromatographic properties (3-6), and in fact NAPE has been positively identified in several plant species (7-12). Reports of labeled NAPE caused us to reexamine the identity of the unknown phospholipid we had isolated previously. In this communication, we report conclusive identification of this phospholipid as *bis*-phosphatidic acid (BPA), the fully acylated analog of phosphatidylglycerol.

MATERIALS AND METHODS

Dicyclohexylcarbodiimide and sodium *bis*(2-methoxyethoxy)-aluminum hydride, 70% in benzene (Vitride®), were purchased from Eastman Organic Chemicals, Rochester, NY. Synthetic tetraoleoyl BPA, egg lecithin phosphatidylglycerol (PG), and beef heart cardiolipin (CL) were from Serdary Research Labora-

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tories, London, Ontario, and standard diglycerides (DG) were obtained from the Hormel Institute Lipids Preparation Laboratory, Austin, MN. N-Palmitoyl PE was synthesized and purified as reported previously (1), pyrophosphatidic acid (PPA) was synthesized by the procedure of Itoh and Kaneko (13), and BPA by the procedure of Baer (14).

Phosphatidic acid (PA) was isolated and purified from soybean phospholipid as described previously (1), but NAPE and the unknown lipid were isolated from soybean phospholipids by an improved thin layer chromatography (TLC) procedure. Total phospholipids (1) were applied to layers of Silica Gel H, 0.5 mm thick; the chromatoplates were developed in chloroform-methanol-7N ammonium hydroxide (75:25:4, v/v/v) and the band just above PE (R_f 0.7), as determined with standards, was scraped off and the lipid was eluted (1). This material was then applied to fresh Silica Gel H plates and developed twice in chloroform-methanol-7N ammonium hydroxide (90:10:1, v/v/v), the first development over one-third of the plate and, after evaporation of the solvent, the second development over the entire plate. Under these conditions, the two compounds were completely separated, NAPE having an R_f of 0.6 and the unknown phospholipid an R_f of 0.4. The fraction from the first Silica Gel H plate could also be separated for analytical purposes on sheets of instant thin layer chromatography-silicic acid (ITLC-SA) (Gelman Instrument Company, Ann Arbor, MI), using a single development in the latter solvent. Natural NAPE was prepared from commercial soybean "lecithin," and the unknown lipid was prepared from the lipids of maturing soybeans, 20-40 days after flowering.

Methanolysis and analysis of methyl esters by TLC and gas liquid chromatography (GLC) were done as reported previously (1). Hydrolysis of phospholipids in 2N HCl at 100 C and ninhydrin tests for amines and amides were performed by the procedure of Dawson et al. (11). Molar ratios of acyl and glycerol were determined by GLC analysis of long chain alcohol acetates and triacetin with internal standards after reduction by Vitride and acetylation as described elsewhere (15). Phosphorus was determined by absorption at 830 nm after acid hydrolysis and treatment with molybdate color reagent (15). Molar ratios of phosphorus: glycerol:acyl were calculated from these data.

Mild alkaline hydrolysis of lipids for deacylation was accomplished by the method of White and Frerman (16), using 0.1 M KOH at 0 C for 2 hr. Products were separated by paper chromatography in phenol-water-acetic acid-ethanol (50:22:3:3, v/v/v/v) (17), and fractions were detected as described by Burrows et al. (18). Acetolysis of lipids in acetic acid-acetic anhydride (3:2, v/v) for 4 hr at 140 C and TLC separation of products in petroleum ether-diethyl ether (4:1, v/v) were by the method of Renkonen (19).
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RESULTS AND DISCUSSION

A novel phospholipid was isolated from immature soybeans. The lipid was more polar than NAPE and could be purified by adsorption TLC; using the procedures described above, about 58 mg of the unidentified phospholipid was obtained from 150 g of immature soybeans. The lipid preparation was pure by ITLC-SA; its migration rate (R_f 0.4) using chloroform-methanol-7N ammonium hydroxide (90:10:1, v/v/v) was identical to that of authentic BPA. In accordance with previous observations (1), the lipid gave a positive phosphomolybdate and negative ninhydrin test both before and after acid hydrolysis (11). Its infrared spectrum exhibited strong absorption bands at 1175 and 1730 cm^{-1} (ester) and 1225 cm^{-1} (P=0), but did not show bands characteristic of amine or amide groups. The spectrum was identical to that of synthetic BPA, similar to those of PG and CL, but distinctly different from that of NAPE which showed a strong band at 1640 cm^{-1} (amide), and of PPA which showed strong absorption at 970 cm^{-1} (P–O–P) and weak absorption at 1060 cm^{-1} (P–O–C). The unidentified lipid had a phosphorus: glycerol:acyl molar ratio of 1:2:4.

Acetolysis (19) of the unidentified lipid followed by TLC fractionation of the reaction products yielded only 1,2-diacyl glycerol acetates. Deacylation by mild alkaline hydrolysis (16) and analysis of the glycerol-phosphate backbone by paper chromatography with a glycerol phosphate (GP) standard revealed that the unidentified lipid yielded a product with the same migration rate (above GP) as that obtained from authentic BPA and PG; the product derived from CL was different (below GP). Thus, the molecular structure of the backbone of the unidentified lipid was G–P–G.

On the basis of these data, we conclude that the unknown lipid heretofore referred to by us as phospholipid X is BPA, the fully acylated analog of phosphatidylglycerol.

Lyso- and semi-lyso-BPA have been found in various mammalian tissues (20-22), including tissues from patients suffering from Niemann-Pick disease (22), in BHK cell cultures (23) and in *Salmonella typhimurium* (24). There have

been relatively few reports on the natural occurrence of the fully acylated phospholipid (BPA), and none in plants. In 1956, Garcia et al. (25) and Olley (26) suggested that a lipid isolated from haddock and cod fish was likely BPA and, more recently, Brotherus and Renkonen (27) reported its presence in cultured BHK cells. In 1975, McAllister and De Siervo (17) demonstrated its presence in the phospholipids of a marine bacterium.

The presence of BPA in immature soybean is of particular interest because it appears to exhibit a relatively high rate of turnover with respect to its fatty acids. Both the amounts of BPA present in the plant tissue and its fatty acid composition change during maturation.

In very young soybeans (20 days after flowering), the lipid fraction consisting of NAPE and BPA can amount to about one-half of the total phospholipids; we found that, at this age, BPA is the major component amounting to 60-80% of this fraction. The relative amounts of both NAPE and BPA apparently decrease substantially during maturation; BPA seems to disappear completely. We were unable to detect BPA in commercial soybean "lecithin" although NAPE could be isolated from this preparation.

The fatty acid composition of BPA from soybeans 20 and 40 days after flowering is given in Table I. Over this time period, the relative amount of linolenic acid in BPA was found to be significantly decreased with a concomitant increase in palmitic acid.

Although our analyses confirmed the presence of NAPE in the soybean, they also showed the presence of a second phospholipid, BPA, possessing very similar chromatographic properties. Singh and Privett (3,4) and Wilson and Rinne (5) reported relatively large amounts of NAPE present during the early stages of soybean development, decreasing with maturation, and also rapid incorporation of radioactivity from labeled lipid precursors into NAPE of developing soybeans. Wilson and Rinne (5) found that freezing of tissues greatly diminished the amount of NAPE which could be extracted. In our experiments, we observed the same characteristics for BPA rather than for NAPE. It is possible that in the studies previously reported incomplete separation resulted in analysis of a mixture of the two phospholipids.

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TABLE I

Fatty Acid Composition (wt %) of BPA of Immature Soybeans 20 and 40 Days After Flowering (DAF)

Fatty acid	BPA (20 DAF)	BPA (40 DAF)
16:0	19	33
18:0	8	8
18:1	6	8
18:2	43	45
18:3	24	6

ACKNOWLEDGMENTS

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[Received February 10, 1977]

The Nomenclature of Lipids[1]

Recommendations (1976)[2,3]

IUPAC-IUB Commission on Biochemical Nomenclature[4]

CONTENTS

In 1967, a "Document for Discussion" on lipid nomenclature (1) was issued by CBN. It included a special system for the designation of configuration in glycerol derivatives that deviated considerably from standard stereochemical nomenclature. This system is based upon a fixed numbering ("stereospecific numbering") for glycerol, regardless of substituents. It was hoped (1) that "discussion will lead shortly to the formulation" of recommendations acceptable to chemists in the field of lipids.

In subsequent years, there has been little discussion about this principle of stereospecific numbering; it has been well-accepted within the field of glycerol derivatives, for which it has been especially useful,[5] and is widely used. However, during this same period, many new and complex lipids and glycolipids have been isolated. Moreover, the Commissions on the Nomenclature of Organic Chemistry (CNOC) and Inorganic Chemistry (CNIC) issued, in 1973, "Nomenclature of Organic Chemistry, Section D" (2), which includes a section on the nomenclature of phosphorus-containing organic compounds and necessitates a reconsideration of the earlier nomenclature (1) in this area.

The present "Recommendations 1976" are based on reports of working groups on lipids[6] and glycolipids.[7] The main features are:

1. the system of stereospecific numbering is retained;
2. semisystematic nomenclature is extended to the plasmalogens;
3. a semisystematic nomenclature for higher glycosphingolipids, based on trivial names for specific tri- and tetrasaccharides, is proposed.

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[1]Document[2] of the IUPAC-IUB Commission on Biochemical Nomenclature (CBN) approved by IUPAC and IUB in 1976, and published with the cooperation of the IUB Commission of Editors of Biochemical Journals.[3] Comments on these Recommendations and suggestions for future revisions may be sent to any member of CBN.[4]

[2]These Recommendations are a revision and extension of "The Nomenclature of Lipids," which appeared in 1967, as amended in 1970 (1).

[3]Reprints of this document, supplied by the publishing journals, may be obtained from W.E. Cohn (Director of the NAS-NRC Office of Biochemical Nomenclature), Biology Division, Oak Ridge National Laboratory, Box Y, Oak Ridge, Tennessee, U.S.A. 37830.

[4]Members of CBN are: O. Hoffmann-Ostenhof (Chairman), W.E. Cohn (Secretary), A.E. Braunstein, H.B.F. Dixon, B.L. Horecker, W.B. Jakoby, P. Karlson, W. Klyne, C. Liébecq, E.C. Webb.

[5]CBN does not wish to imply that the idea of stereospecific numbering should be applied to other groups of compounds. It is the symmetry of glycerol itself, but the asymmetry of its derivatives carrying different substituents at *O*-1 and *O*-3, as well as the unique place of these compounds in lipid metabolism, that makes this special treatment desirable.

[6]Members of the Working Group on Lipid Nomenclature: H. Hirschmann (U.S.A.), P. Karlson (Germany; convenor), W. Stoffel (Germany), F. Snyder (U.S.A.), S. Veibel (Denmark), F. Vögtle (Germany).

[7]Members of the Working Group on Glycolipid Nomenclature: S. Basu (U.S.A.), R.O. Brady (U.S.A.), R.M. Burton (U.S.A.), R. Caputto (Argentina), S. Gatt (Israel), S.I. Hakomori (U.S.A.), M. Philippart (U.S.A.), L. Svennerholm (Sweden), D. Shapiro (Israel), C.C. Sweeley (U.S.A.), H. Wiegandt (Germany; convenor).

RECOMMENDATIONS

I. FATTY ACIDS, NEUTRAL FATS, LONG-CHAIN ALCOHOLS AND LONG-CHAIN BASES

A. Generic Terms

Lip 1.1 The term "**fatty acid**" designates any one of the aliphatic monocarboxylic acids that can be liberated by hydrolysis from naturally occuring fats and oils. In the terms "free fatty acids" or "nonesterified fatty acids," now widely in use, "free" and "nonesterified" are actually redundant and should be omitted (See Lip. 1.14). [The designation "aliphatic carboxylate (C_{10}-C_{26}, nonesterified)" used by the Commission on Quantities and Units in Clinical Chemistry is correct, but rather cumbersome.] Whenever the *sum* of fatty acids and their esters is determined by an analytical method, this should be explicitly stated. (See also Lip 1.6).

Lip 1.2 "**Neutral fats**" are mono-, di-, or triesters of glycerol with fatty acids, and are therefore termed monoacylglycerol, diacylglycerol, or triacylglycerol, as appropriate. "Acylglycerols" includes mixtures of any or all of these.

Comments. i) The term "acyl" is used in Organic Nomenclature (3) to denote the radical formed by loss of the OH group from the acid function of any acid (cf. Lip 1.6). We are concerned here with acyl radicals of aliphatic carboxylic acids with four or more carbon atoms, the larger members of which ($>C_{10}$) are also known as "higher fatty acids."

ii) The old terms monoglyceride, diglyceride, and triglyceride are discouraged and should progressively be abandoned, not only for consistency, but mainly because strict interpretation does not convey the intended meaning. "Triglyceride," taken literally, indicates three *glycerol* residues (e.g., cardiolipin), diglyceride two (e.g., phosphatidylglycerol), and a monoglyceride is a mono*acyl*glycerol.

Lip 1.3 The generic term "long-chain alcohol" or "fatty alcohol" refers to an aliphatic compound with a chain-length greater than C_{10} that possesses a terminal CH_2OH group. Such alcohols should be named according to systematic nomenclature (3). (See Lip 1.7).

Lip 1.4 The term "**sphingoid**" or "sphingoid base" refers to sphinganine (cf. Lip 1.8), [D-*erythro*-2-amino-1,3-octadecanediol (I)], to its homologs and stereoisomers (II,III), and to the hydroxy and unsaturated derivatives of these compounds (IV-VI). The term "long-chain base" may be used in a wider sense to indicate any base containing a long-chain aliphatic radical.

Lip. 1.5 The following generic terms are used for the following groups of compounds:

(a) **sphingolipid**, for any lipid containing a sphingoid;
(b) **ceramide**, for an *N*-acylated sphingoid;
(c) **sphingomyelin**, for a ceramide-1-phosphocholine. (See ref. 2 for this use of "phospho"; also Lip 2.11);
(d) **glycosphingolipid**, for any lipid containing a sphingoid and one or more sugars. (See sections I,B,2 and II,A for other generic terms).

B. Individual Compounds

1. Fatty acids and alcohols.

Lip 1.6 Fatty acids (cf. Lip 1.1) and their acyl radicals (cf. Lip 1.2, comment i) are named according to the IUPAC Rules for the Nomenclature of Organic Chemistry (ref. 3, Rule C-4). (A list of trivial names is given in Appendix A.) Fatty acids are numbered with the carbon atom of the carboxyl group as C-1. By standard biochemical convention, the ending "-ate" in, e.g., palmitate denotes any mixture of the free acid and its ionized form in which the cations are not specified. The ending "-ate" is also used to designate esters, e.g., cholesteryl palmitate, ethylidene dilaurate, etc. (cf. Lip 1.12). Structural isomers of polyunsaturated acids, hitherto distinguished by Greek letters (e.g., α- and γ-linolenic acids), are better distinguished by the locants of the unsaturated linkages [e.g., (9,12,15)- and (6,9,12)-linolenic acids, respectively]. (See Lip 1.15). However, the Greek letter prefixes may be useful in (defined) abbreviations (see Appendix A).

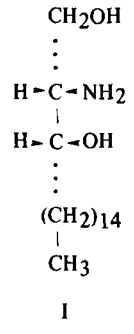
Lip 1.7 Long-chain alcohols (fatty alcohols) and the radicals derived from them should be designated by their systematic names (ref. 3, Rules C-201 and A-1 et seq.), but not by trivial names that are derived from those of fatty acids.

Examples:

1-hexadecanol and hexadecyl-, not palmityl alcohol and palmityl-;
1-dodecanol and dodecyl-, not lauryl alcohol and lauryl-.

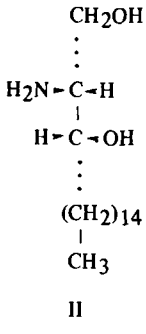
2. Sphinganine and derivatives.

Lip 1.8 The compound previously known as dihydrosphingosine [2*D*-amino-1,3*D*-octadecanediol or D-*erythro*-2-amino-1,3-octadecanediol or (2*S*, 3*R*)-2-amino-1,3-octadecanediol] is called **sphinganine** (I).



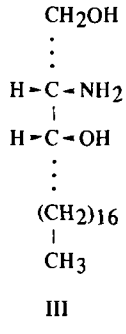
I

Sphinganine [*D-erythro* or 2*S*,3*R* configuration implied]



II

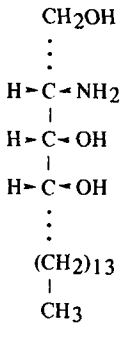
(2*R*,3*R*)- (or D-*threo*-) -2-Amino-1,3-octadecanediol



III

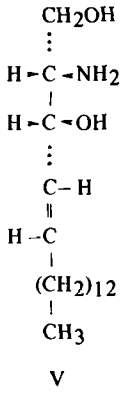
Icosasphinganine*

*Formerly eicosasphinganine (see footnote [d] in Appendix A)



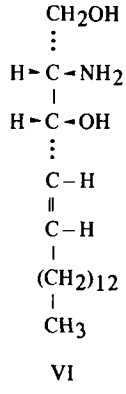
IV

4*D*-Hydroxysphinganine; (2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol; (phytosphingosine).



V

Sphingosine; (4*E*)-sphingenine; *trans*-4-sphingenine; (2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol;



VI

cis-4-Sphingenine; (4*Z*)-sphingenine.

Lip 1.9 Trivial names of higher or lower homologs of sphinganine may be derived by adding a prefix (ref. 3, Rule A-1) denoting the total number of carbon atoms in the main chain of the homolog, e.g., icosasphinganine* for the C_{20} compound (III), hexadecasphinganine for the C_{16} compound.

Lip 1.10 Affixes denoting substitution of sphinganine (hydroxy, oxo, methyl, etc.) are used as usual, according to existing rules (3). The configurations of additional substituents should be specified by the prefixes "*D*" or "*L*" (italic capitals, cf. ref. 4) following the locant of substitution. These prefixes refer to the orientation of the functional groups to the right or left, respectively, of the carbon chain when written vertically in a Fischer projection with C-1 at the top (cf. Formulae I-VI). If the configuration is unknown, the prefix "*X*" may be used. In the case of a racemic mixture, "*rac*" should be used as a prefix to the name.

Sphingoids differing from sphinganine in their configurations at C-2 and/or C-3 should be named *not* as derivatives of sphinganine, but with fully systematic names (3), using the prefixes D-*threo*, L-*erythro*, as appropriate, e.g., D-*threo*-2-amino-1,3-octadecanediol, or (2*R*, 3*R*)-2-amino-1,3-octadecanediol, for II (cf. Rule Carb-8 in ref. 5). (Cf. Lip 1.11, ex. 4).

Comments. i) The semisystematic names for the sphingoids are significantly shorter than the fully systematic names only if the terms chosen imply not only substituents but also configurations. Therefore, the name "sphinganine" specifies the D-*erythro* configuration, and it is

*See footnote d in Appendix A re "icosa" for "eicosa."

logical that the names of stereoisomers of sphinganine differing in configuration at C-2 and/or C-3 should not include "sphinganine" as a root. [This recommendation differs from that in the previous document (1).]

ii) The configurations usually encountered have identical configurational prefixes only if a *D/L* but not if the *R/S* system (6) is used; e.g., C-3 is *D* and *R* in icosasphinganine (III) and *D* and *S* in 4*D*-hydroxysphinganine (IV). Whenever it is desirable to use the *R/S* system, the fully systematic name should be used with the specification of configuration at every center (and, when applicable, of the configuration at the double bond).

Examples:

(2*R*,3*R*)-2-amino-1,3-octadecanediol, for II;
(2*S*,3*S*,4*R*)-2-amino-1,3,4-octadecanetriol for IV;
(2*S*,3*R*,4*E*)-2-amino-4-octadecene-1,3-diol for sphingosine (V) (See also Lip 1.11).

Lip 1.11 Names for unsaturated compounds are derived from the names of the corresponding saturated compounds by the appropriate infixes, namely ene, diene, yne, etc. (3). If the geometry of the double bond is known, it should be indicated by the more modern *E-Z* system (cf. 6, Rule E-2.2), e.g., (4*E*)-sphingenine for sphingosine (V).

Comment. The trivial name "**sphingosine**" (V) is retained. If trivial names other than sphingosine are used, they should be defined in each paper in terms of this nomenclature, or of the general nomenclature of organic chemistry (3).

Other names for compounds described in Lip 1.10 and 1.11
4*D*-hydroxysphinganine for IV, formerly known as phytosphingosine;
(4*E*)-sphingenine for sphingosine (V);
(4*Z*)-sphingenine for the geometric isomer of sphingosine (VI);
D-*threo*-2-amino-1,3-octadecanediol for the C-2 epimer of sphinganine (II); cf. Lip. 1.10, Ex. 1.

3. Glycerol derivatives

Lip 1.12 Esters, ethers and other *O*-derivatives of glycerol are designated according to Carb-15 of the Rules of Carbohydrate Nomenclature (5), i.e. by a prefix, denoting the substituent, preceded by a locant. If the substitution is on a carbon atom, the compound is designated by its systematic name and not as a derivative of glycerol (e.g., 1,2,3-nonadecanetriol for $C_{16}H_{33}CHOH\text{-}CHOH\text{-}CH_2OH$, which could be considered as 1-*C*-hexadecylglycerol). It is permissible to omit the locant "*O*" if the substitution is on the oxygen atoms of glycerol.

Examples:

tristearoylglycerol or tri-*O*-stearoylglycerol or glycerol tristearate, or glyceryl tristearate;
1,3-benzylideneglycerol or 1,3-*O*-benzylideneglycerol;
glycerol 2-phosphate (a permissible alternative to this term is 2-phosphoglycerol) (10).

Comment. The alternative system set forth in Carb-16 of the Rules on Carbohydrate Nomenclature (5), i.e. the use of the suffix "-ate," is less suitable for glycerol esters, with the exception of the phosphates (see *Examples*). However, this system may be used to designate esters of monofunctional alcohols, e.g. cholesteryl palmitate (cf. Lip 1.6).

Lip 1.13 **Stereospecific numbering.** In order to designate the configuration of glycerol derivatives, the carbon atoms of glycerol are numbered stereospecifically. The carbon atom that appears on top in that Fischer projection that shows a vertical carbon chain with the hydroxyl group at carbon-2 to the left is designated as C-1. To differentiate such numbering from conventional numbering conveying no steric information, the prefix "*sn*" (for *stereospecifically numbered*) is used. This term is printed in lower-case italics, even at the beginning of a sentence, immediately preceding the glycerol term, from which it is separated by hyphen. The prefix "*rac-*" (for *racemo*) precedes the full name if the product is an equal mixture of both antipodes; the prefix "*X-*" may be used when the configuration of the compound is either unknown or unspecified (cf. Lip 1.10).

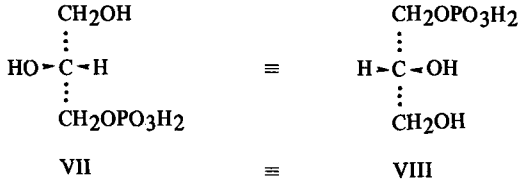
Examples:

sn-glycerol 3-phosphate for the stereoisomer (VII≡VIII), previously known as either L-α-glycerophosphate or as D-glycerol 1-phosphate;
rac-1-hexadecylglycerol;
1,2-dipalmitoyl-3-stearoyl-*X*-glycerol

Comments. i) The problem of distinguishing between stereoisomers was discussed in extenso in the 1967 document (1). In brief, difficulties arise because glycerol is a prochiral compound. The parent substance of many phospholipids, natural glycerol phosphate, has been named both as L-α-glycerol phosphate (7) (VII) and, according to standard rules of nomenclature, D-glycerol 1-phosphate (8) (VIII). When the *R/S* system (sequence rule) is applied, substitution of one of the primary hydroxyl groups often leads to changes in the configurational prefix, thus obscuring chemical and biogenetical relationships; it is generally inapplicable to the steric description of such mixtures as

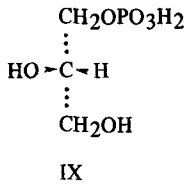
occur in triacylglycerols isolated from natural sources. The stereospecific numbering of glycerol and its derivatives as proposed by Hirschmann (9), described above and in (1), avoids these difficulties; it has proved useful and is widely accepted.

ii) The enantiomer of *sn*-glycerol 3-phosphate (VII) is *sn*-glycerol 1-phosphate (IX), as is evident from the structures.



VII ≡ VIII

sn-Glycerol 3-phosphate
[L-(glycerol 3-phosphate) ≡ D-(glycerol 1-phosphate)]



IX

sn-Glycerol 1-phosphate
[L-(glycerol 1-phosphate) ≡ D-(glycerol 3-phosphate)]

C. Symbols and Abbreviations

Lip. 1.14 The term "fatty acids" (cf. Lip 1.1) should not be abbreviated. The use of abbreviations like "FFA" for " free fatty acids" or "NEFA" for "nonesterified fatty acids" is strongly discouraged.

Comment. The words "acids" and "esters" serve to distinguish the "free" (nonesterified) and "bound" (esterified) fatty acids and are as short or shorter than the abbreviations themselves.

Lip 1.15 In tables and discussions where various fatty acids are involved, the notation giving the number of carbon atoms and of double bonds (separated by a colon) is acceptable, e.g. 16:0 for palmitic acid, 18:1 for oleic acid. Also, terms such as "(18:0)acyl" may be used to symbolize radicals of fatty acids. (See Appendix A).

Comment. This system is already widely used. It should, however, be kept in mind that it sometimes does not completely specify the fatty acid. For example, α-linolenic acid and γ-linolenic acid are both 18:3 acids; the designation 18:3 is therefore ambiguous. In such a case, the position of double bonds should be indicated, e.g. 18:3(9,12,15) for (9,12,15)-linolenic acid, formerly known as α-linolenic acid.

Lip 1.16 It is sometimes desirable (for example, in discussing the biosynthesis of lipids) to indicate the position of each double bond with reference not to the carboxyl group (always C-1), but to the end of the chain remote from the carboxyl. If *n* is the number of carbon atoms in the chain (i.e., the locant of the terminal methyl group) and *x* is the (lower) locant of the double bond, the position of the double bond may be defined as (*n* minus *x*). Thus, the common position of the double bond in oleic and nervonic acids may be given as 18-9 and 24-9, respectively. This structural regularity should not be expressed as ω9.

Lip 1.17 The system described in Lip 1.15 may also be used to denote alcohols and aldehydes related to fatty acids, provided that the nature of the residue is clearly indicated either by the appropriate name of the compound(s) (e.g. 18:1 alcohol) or in the heading of the table. The 1-ene of alk-1-en-1-yl (i.e., 1-alkenyl) compounds is not counted in this system (see Lip 2.10, comment).

Lip 1.18 For many complex lipids, a representation of the structures using symbols rather than structural formulae may be useful. Symbols proposed for various constituents are given in Appendix B (see also ref. 10), and, for glycolipids, in Lip 3.13. They are constructed in analogy to those in use for amino acids (11), nucleosides (12) and saccharides (13).

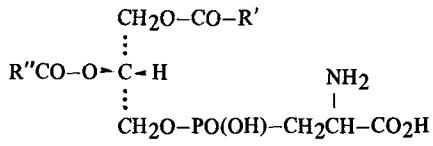
II. PHOSPHOLIPIDS

A. Generic Terms

The Rules of The Nomenclature of Organic Phosphorus Compounds (also known as D-Rules) (2) recognize, for biochemical usage, the prefix "phospho-" as an alternate to "*O*-phosphono-" (or "*N*-phosphono-"). By a similar convention (10), "-phospho-" may be used as an infix to designate the phosphodiester bridge present in such compounds as glycerophosphocholine. The use of root names like "phosphatidic acid" is retained and extended (Lip 2.3-2.4).

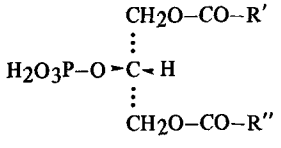
Lip 2.1 "**Phospholipid**" may be used for any lipid containing phosphoric acid as mono- or diester. Likewise, lipids containing *C*-phosphono groups (e.g. compound X) may be termed "phosphonolipids."

Lip 2.2 "**Glycerophospholipid**" signifies any derivative of glycerophosphoric acid that con-



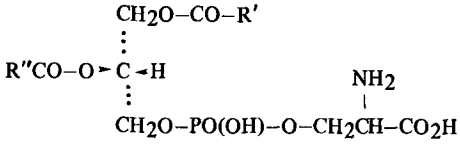
X

A phosphonolipid



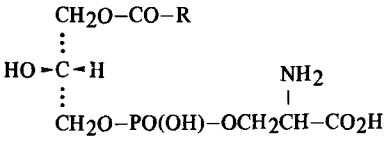
XI

2-Phosphatidic acid



XIIa

Phosphatidylserine
(3-*sn*-phosphatidylserine)



XIIb

A 2-lysophospholipid

tains at least one *O*-acyl, or *O*-alkyl, or *O*-(1-alkenyl) group attached to the glycerol residue. Generic names for other classes of phospholipids may be coined according to this scheme, e.g., **sphingophospholipid, inositolphospholipid.**

Comment. The old terms, "phosphatide," "phosphoglyceride," and "phosphoinositide" are no longer recommended because they do not convey the intended meaning (see also Lip 1.2).

Lip 2.3 "**Phosphatidic acid**" signifies a derivative of a glycerol phosphate (glycerophosphate) in which both remaining hydroxyl groups of glycerol are esterified with fatty acids. The position of the phosphate group may be emphasized by stereospecific numbering.

Comment. For the most common (3-*sn*) phosphatidic acid and its derivatives, the locants are often omitted. However, "phosphatidyl" without locants can lead to ambiguities. It is therefore preferable to use the proper locants, for example, 2-phosphatidic acid for compound XI, and 3-*sn*-phosphatidylserine for XIIa.

Lip 2.4 The common glycerophospholipids are named as derivatives of phosphatidic acid, e.g. 3-*sn*-phosphatidylcholine (this term is preferred to the trivial name, lecithin; the systematic name is 1,2-diacyl-*sn*-glycero-3-phosphocholine); 3-*sn*-phosphatidylserine; 1-phosphatidylinositol (see comment ii below); 1,3-bis(3-*sn*-phosphatidyl)glycerol.

Comments. i) It is understood that, in combination with compounds like ethanolamine (properly, 2-aminoethanol) or serine, which bear both hydroxyl and amino groups, substitution by phosphorus is at the hydroxyl group of the ethanolamine or serine. Substitution at any other position, or where confusion may arise, requires a locant.

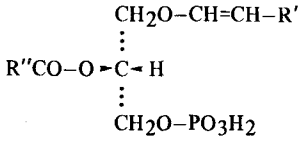
ii) The phosphorylated derivatives of 1-(3-*sn*-phosphatidyl)inositol should be called 1-phosphatidylinositol 4-phosphate and 1-phosphatidylinositol 3,4-bisphosphate, respectively. The use of "diphosphoinositide" and "triphosphoinositide" for these is discouraged, as these names do not convey the chemical structures of the compounds and can be misleading. [Cf. also Table IV in (10).]

Lip 2.5 As an alternative, generic names may be coined according to Lip 1.13, i.e. using glycerol phosphate (glycerophosphate) as the stem. In this case, the stereospecific numbering of glycerol should be used to indicate the position of the phosphoric residue as well as the other substituents (acyl-, alkyl-, 1-alkenyl). If the nature of these substituents cannot be specified, the prefix "radyl" may be used.

Lip 2.6 Derivatives of phosphatidic acids resulting from hydrolytic removal of one of the two acyl groups may be designated by the old prefix "**lyso**," e.g. lysophosphatidylethanolamine for compound XIIb. A locant may be added to designate the site of (hydro)lysis, 2-lyso designating hydrolysis at position 2, leaving a free hydroxyl group at this carbon atom.

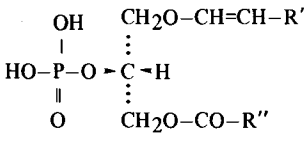
Comment. The "lyso" term originated from the fact that these compounds are hemolytic. It is here redefined to indicate a limited hydrolysis of the phosphatidyl derivative (i.e., "deacyl").

Lip 2.7 The term "**plasmalogen**" may be used as a generic term for glycerophospholipids in which the glycerol moiety bears an 1-alkenyl ether group.



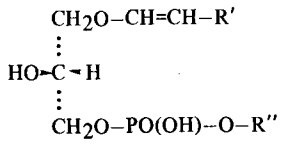
XIII

A plasmenic acid



XIV

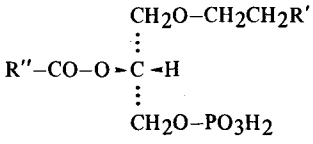
3-Acyl-1-(1-alkenyl)-*sn*-glycerol 2-phosphate



XV

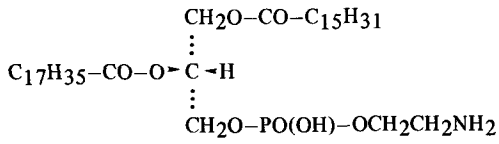
XVa: (R″ = H): a lysoplasmenic acid

XVb: (R″ = $CH_2CH_2NH_2$) a lysoplasmenylethanolamine



XVI

A plasmanic acid



XVII

1-Palmitoyl-2-stearoyl-*sn*-glycero-3-phosphoethanolamine

Lip 2.8 The term "**plasmenic acid**" signifies a derivative of *sn*-glycero-3-phosphate in which carbon-1 bears an *O*-(1-alkenyl) residue, and position 2 is esterified with a fatty acid (XIII). This term can also be used to name derivatives, e.g. plasmenylethanolamine.

Comments. i) The use of "phosphatidyl" as a name for the acyl radical of phosphatidic acid has facilitated the nomenclature of its various compounds (see Lip 2.4). Therefore, it seems logical to offer a similar short term for XIII, i.e., "plasmenic acid," as an alternative to the more systematic name, 2-acyl-1-alkenyl-*sn*-glycerol 3-phosphate, which, of course, may be used if desired. "Plasmenic" is a contraction of "plasmalogenic" and may be especially useful in naming derivatives, e.g., plasmenylserine.

ii) Isomers like those bearing the phosphate residue in position 2 (e.g. compound XIV) should not be named in this way but as derivatives of the corresponding glycerophosphate, using stereospecific numbering.

Lip 2.9 The term "**lysoplasmenic acid**" may be used for a derivative of *sn*-glycero-3-phosphate that has an *O*-(1-alkenyl) residue on carbon-1, the hydroxyl group in position 2 being unsubstituted (XVa). This name may also be used in combinations like "lysoplasmenylethanolamine" (XVb).

Lip 2.10 For compounds of type XVI, bearing a saturated ether group in position 1 and an acyl group in position 2 of *sn*-glycero-3-phosphate, the term "**plasmanic acid**" is proposed. Compounds deacylated in position 2, or with a substituent on the phosphoric residue, can be treated as are the plasmenic acids (Lip 2.9).

Comment. The proposed names will be especially useful for naming phosphoric diesters (phosphodiesters), e.g., plasmanylethanolamine, instead of 2-acyl-1-alkyl-*sn*-glycero-3-phosphoethanolamine. The terms "plasmanic acid" and "plasmanyl" may also be applied to ethers with an alkyl group bearing a double bond within the chain, e.g. a 9-hexadecenyl residue (derived from palmitoleic acid). In such cases, the proper term "alkenyl," if used without the ene locant(s), would be misleading. (See Lip 1.17).

B. Individual compounds

Lip 2.11 Individual glycerophospholipids in which the substituents can be specified are named according to existing Rules (2,3,5,6), using the infix "-phospho-" (2,10) to indicate the phosphodiester bridge.

Example:

1-Palmitoyl-2-stearoyl-*sn*-glycero-3-phosphoethanolamine for compound XVII.

Lip 2.12 The ketone derived from glycerol, 1,3-dihydroxy-2-propanone, also known as dihydroxyacetone, may be termed "glycerone," if desired. The name is a contraction of "glyceroketone" and may be useful to emphasize the relationship with glycerol, glyceraldehyde (glyceral), and glycerate. It also permits a simple symbolism (Appendix B) and the naming of derived lipids, e.g., 1-palmitoyl-3-phosphoglycerone.

III. GLYCOLIPIDS

A. General Considerations

Glycolipids (a contraction of glycosyllipids) are generally named as glycosyl derivatives of the corresponding lipid, e.g., diacylgalactosylglycerol, glucosylceramide. Because of the heterogeneity of the fatty acids and long-chain bases encountered in most cases, a generic name for the lipid moiety is needed, i.e. ceramide. With higher glycosphingolipids, especially the gangliosides, naming problems arise from the complexity of the carbohydrate moiety of these compounds. The systematic names of the oligosaccharides linked to ceramide are so cumbersome that they are of the same practical value as, e.g., the systematic name for a peptide hormone such as insulin. It was felt that this difficulty could be overcome only by creating suitable trivial names for some parent oligosaccharides. In constructing these names (see Table I) the following principles were observed:

1. The number of monosaccharide units in an oligosaccharide is indicated by the suffixes "-biose," "-triaose," "-tetraose," etc. This follows the well-established practice in the carbohydrate field (cf. cellobiose, cellotetraose, maltotetraose, etc.), with the exception that the suffix "-triose," as used in maltotriose, has been changed to "-triaose" to avoid confusion with the monosaccharides called trioses.

2. The oligosaccharides are grouped in series according to their structure and biogenetic relationship.

3. Differences in linkage (e.g., 1→4 versus 1→3) in otherwise identical sequences are indicated by "iso-" or "neo-," used as a prefix. On the basis of these names, the semisystematic nomenclature for neutral glycosphingolipids and gangliosides described below is recommended. A set of symbols has been devised that allows a simple representation of complex neutral and acidic glycosphingolipids (Table I).

B. Generic Terms

Lip 3.1 The term "**glycolipid**" designates any compound containing one or more monosaccharide residues linked by a glycosyl linkage to a lipid part [e.g., a mono- or diacylglycerol, a long-chain base (sphingoid) like sphingosine, or a ceramide].

Lip 3.2 The term "**glycoglycerolipid**" may be used to designate glycolipids containing one or more glycerol residues.

Lip 3.3 The term "**glycosphingolipid,**" as hitherto, includes all compounds containing at least one monosaccharide and a sphingoid. The glycosphingolipids can be subdivided as follows:

Neutral glycosphingolipids:
Monoglycosyl- and oligoglycosylsphingoids; monoglycosyl- and oligoglycosylceramides.
Acidic glycosphingolipids:
sialosylglycosylsphingolipids (gangliosides); sulfoglycosylsphingolipids (formerly "sulfatides," which is not recommended) (cf. Lip 3.11).

Lip 3.4 "**Psychosine**" may be used as a generic name for 1-monoglycosylsphingoids, although the latter is preferred. The nature of the monosaccharide and the sphingoid is not specified in this name.

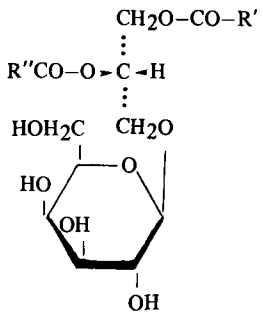
Lip 3.5 The term "**fucolipid**" may be used to designate fucose-containing neutral or acid glycolipids.

C. Individual Compounds*

Lip 3.6 Glycoglycerolipids may be named either as glycosyl compounds according to Rule Carb-24 or as glycosides according to Rule Carb-23 (5).

Example:

Compound XVIII may be named either 1,2-diacyl-3-β-D-galactosyl-*sn*-glycerol or 1,2-diacyl-*sn*-glycerol 3-β-D-galactoside.



XVIII

Comment. The first form is preferred, as the glycosphingolipids are also named this way.

Lip 3.7 A glycosphingolipid is generally named as a "**glycosylsphingoid**" or a "**glycosylceramide,**" using the appropriate trivial name of the mono- or oligosaccharide residue for "glycosyl." It is understood that the sugar residue is attached to the C-1 hydroxyl group of the ceramide. For glycosphingolipids carrying two to four saccharide residues, the trivial names listed in Table I are recommended.

*"Individual" in this section refers to the carbohydrate moiety only.

Comment. It is strongly recommended that the name of the oligosaccharide be defined in each publication by means of the standard symbols for sugars (as in Table I, column 1) rather than by the full name, which is often so long as to be confusing.

Lip 3.8 The trivial name **"cerebroside"** designates 1-β-glycosylceramide (the natures of the sphingoid and of the fatty acid are not specified in this name).

Lip 3.9 Glycosphingolipids carrying fucose either as a branch or at the end of an oligohexosylceramide are named as "fucosyl(*X*)osylceramide" where (*X*) stands for the root name of the oligosaccharide. The location of the fucosyl residue is indicated by a Roman numeral designating the position of the monosaccharide residue in the parent oligosaccharide (counting from the ceramide end) to which the fucose residue is attached, with an Arabic numeral superscript indicating the position within that residue to which the fucose is attached. If necessary, the anomeric symbol can be used as usual, i.e., preceding "fucosyl-."

Examples for Lip 3.7 and Lip 3.9 (structures given in the symbols of Lip 3.13):**

Lactosylceramide for Gal (β1→4)GlcCer;
mucotriaosylceramide for
Gal(β1→4)Gal(β1→4)GlcCer;
III^2-α-fucosylisoglobotriaosylceramide for
Fuc(α1→2)Gal(α1→3)Gal(β1→4)Glc(β1→1)Cer.

Lip 3.10 **Sialoglycosphingolipids** (synonym: **gangliosides**) are glycosphingolipids carrying one or more sialic residues. **Sialic acid** is the generic term for *N*-acetyl- or *N*-glycoloylneuraminic acid (cf. section 3 in ref. 1). Gangliosides are named as *N*-acetyl- (or *N*-glycoloyl-) neuraminosyl-(*X*)osylceramide, where (*X*) stands for the root name of the neutral oligosaccharide to which the sialosyl residue is attached (cf. Table I). The position of the sialosyl residue is indicated in the same way as in the case of fucolipids (see Lip 3.9).

Example:

II^3-*N*-acetylneuraminosyllactosylceramide for
AcNeu(α2→3)Gal(β1→4)Glc(β1→1)Cer

Lip 3.11 Glycosphingolipids carrying a sulfuric ester (sulfate) group, formerly called sulfatides, are preferably named as sulfates of the parent neutral glycosphingolipid. The location of the sulfate group may be indicated as in Lip. 3.9.

Example:

lactosylceramide II^3-sulfate

Lip 3.12 Phosphoglycosphingolipids with phosphodiester structures are named according to the recommendation for the phospholipids (see Section II).

D. Symbols and Abbreviations.

Lip 3.13 Simple or complex glycosphingolipids can be represented according to existing rules, using the symbols Cer, Sph, AcNeu, etc. (Appendix B), together with the recommended (13) symbols for the hexoses (Glc, Gal, etc.). Examples are given above, and in Table I and Appendix C. However, due to the complexity of the higher glycosphingolipids, this often results in very long and cumbersome series that are not easy to comprehend. *It is therefore recommended* that the oligosaccharides listed in Table I be represented by specific symbols in which the number of monosaccharide units (-oses) is indicated by Ose_n, preceded by two letters representing the trivial name of the oligosaccharide (column 3). For a short form, which may be required in the case of limited space or frequent repetition, Ose can be omitted (column 4); however, the long form is preferred as being more evocative.

Examples:

(i) $McOse_3Cer$ for mucotriaosylceramide,
Gal(β1-4)Gal(β1-4)Glc(1-1)Cer;
(ii) II^{3}AcNeu-$GgOse_4Cer$ for II^3-*N*-acetylneuraminosylgangliotetraosylceramide,
Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc)β1→4Glcβ1→1Cer
(see Lip 3.14 for this mode of representing a branched chain.).

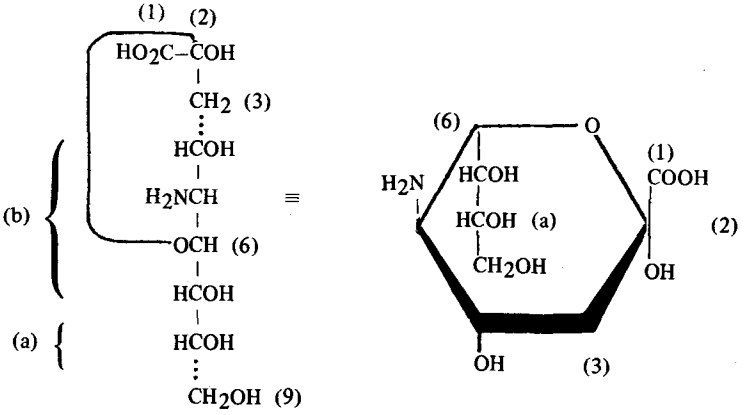
Abbreviations for the more important gangliosides are given in Appendix C.

Lip 3.14 When it is desirable to represent a branched oligosaccharide on a single line, as in running text or a table, the parentheses surrounding the locants in the main chain may be omitted and used instead to enclose the symbols for the branched portion(s) of the molecule. The branches follow, in parentheses and with appropriate arrows, the residues to which they are attached.

Examples:

(i) NeuGcα2→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuGc)β1→4Glcβ1→1Cer;
(ii) NeuAcα2→3Galβ1→3GalNAcβ1→4Gal(3←2αNeuAc8←2αNeuAc)β1→4Glcβ1→1Cer
(iii) GalNAcα1→3Gal(2←1αFuc)β1→4GlcNAc(3←1αFuc)β1→3Galβ1→4Glcβ1→1Cer
≡ III^3,IV^2-α,α-Difucosyl-IV^3-α-2-acetamido-2-deoxygalactosylneolactotetraosylceramide
≡ III^{3}IV^2(Fucα)$_2$,IV^{3}GalNAcα-n$LcOse_4$Cer.

**D is omitted by convention in the abbreviated formulas, but D (or L) may be inserted when desirable. Hyphens may replace left-to-right arrows (see section 3.4 of ref. 13).



XIX

Neuraminic acid

5-Amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulopyranosonic acid
(a) (b)

IV. NEURAMINIC ACID

Lip 4.1 The compound 5-amino-3,5-dideoxy-D-*glycero*-D-*galacto*-nonulosonic acid is **neuraminic acid** (XIX), with the symbol Neu (11).

Lip 4.2 The term "sialic acid" signifies the N-acylneuraminic acids and their esters and other derivatives of the alcoholic hydroxyl groups.

Lip. 4.3 The radicals resulting from the removal of a hydroxyl group of neuraminic acid or sialic acid are designated as **neuraminoyl** or **sialoyl**, respectively, if the hydroxyl is removed from the carboxyl group, and as **neuraminosyl** and **sialosyl**, respectively, if the hydroxyl group is removed from the anomeric carbon atom of the cyclic structure.

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TABLE I

Names and Abbreviations of Simple Glycolipids

Structure[a]	Trivial name of Oligosaccharide[b]	Symbol[c]	Short symbol[d]
Gal(α1-4)Gal(β1-4)GlcCer	Globotriaose	$GbOse_3$	Gb_3
GalNAc(β1-3)Gal(α1-4)Gal(β1-4)GlcCer	Globotetraose	$GbOse_4$	Gb_4
Gal(α1-3)Gal(β1-4)GlcCer	Isoglobotriaose	$iGbOse_3$	iGb_3
GalNAc(β1-3)Gal(α1-3)Gal(β1-4)GlcCer	Isoglobotetraose	$iGbOse_4$	iGb_4
Gal(β1-4)Gal(β1-4)GlcCer	Mucotriaose	$McOse_3$	Mc_3
Gal(β1-3)Gal(β1-4)Gal(β1-4)GlcCer	Mucotetraose	$McOse_4$	Mc_4
GlcNAc(β1-3)Gal(β1-4)GlcCer	Lactotriaose	$LcOse_3$	Lc_3
Gal(β1-3)GlcNAc(β1-3)Gal(β1-4)GlcCer	Lactotetraose	$LcOse_4$	Lc_4
Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcCer	Neolactotetraose	$nLcOse_4$	nLc
GalNAc(β1-4)Gal(β1-4)GlcCer	Gangliotriaose	$GgOse_3$	Gg_3
Gal(β1-3)GalNAc(β1-4)Gal(β1-4)GlcCer	Gangliotetraose	$GgOse_4$	Gg_4
Gal(α1-4)GalCer	Galabiose	$GaOse_2$	Ga_2
Gal(1-4)Gal(α1-4)GalCer	Galatriaose	$GaOse_3$	Ga_3
GalNAc(1-3)Gal(1-4)Gal(α1-4)GalCer	*N*-Acetylgalactosaminylgalatriaose	$GalNAc1\text{-}3GaOse_3$	–

[a]Symbols and arrangement are discussed in Lip 3.13. Hyphens replace left-to-right arrows (see Section 3.4 of ref. 13).

[b]Name of glycolipid is formed by converting ending "-ose" to "-osyl," followed by "-ceramide," without space; e.g., globotriaosylceramide.

[c]Should be followed by Cer for the glycolipid, without space; e.g., $McOse_3Cer$, Mc_4Cer (see Lip 3.13).

[d]The short form should be used only in situations of limited space or in case of frequent repetition.

APPENDIX A

Names of and Symbols for Higher Fatty Acids

	Numerical Symbol	Structure $H_3C-(R)-CO_2H$	Stems of Systematic Names[a]	Stems of Trivial Names[b]	"Name" Symbol
1	10:0	$-(CH_2)_8-$	Decano-	Capr-[c]	Dec
2	12:0	$-(CH_2)_{10}-$	Dodecano-	Laur-	Lau
3	14:0	$-(CH_2)_{12}-$	Tetradecano-	Myrist-	Myr
4	16:0	$-(CH_2)_{14}-$	Hexadecano-	Palmit-	Pam
5	16:1	$-(CH_2)_5CH=CH(CH_2)_7-$	9-Hexadeceno-	Palmitole-	ΔPam
6	18:0	$-(CH_2)_{16}-$	Octadecano-	Stear-	Ste
7	18:1(9)	$-(CH_2)_7CH=CH(CH_2)_7-$	*cis*-9-Octadeceno-	Ole-	Ole
8	18:1(11)	$-(CH_2)_5CH=CH(CH_2)_9-$	11-Octadeceno-	Vaccen-	Vac
9	18:2(9,12)	$-(CH_2)_3(CH_2CH=CH)_2(CH_2)_7-$	*cis,cis*-9,12-Octadecadieno-	Linole-	Lin
10	18:3(9,12,15)	$-(CH_2CH=CH)_3(CH_2)_7-$	9,12,15-Octadecatrieno-	(9,12,15)-Linolen-	αLnn
11	18:3(6,9,12)	$-(CH_2)_3(CH_2CH=CH)_3(CH_2)_4-$	6,9,12-Octadecatrieno-	(6,9,12)-Linolen-	γLnn
12	18:3(9,11,13)	$-(CH_2)_3(CH=CH)_3(CH_2)_7-$	9,11,13-Octadecatrieno-	Eleostear-	eSte
13	20:0	$-(CH_2)_{18}-$	Icosano[d]	Arachid-	Ach
14	20:2(8,11)	$-(CH_2)_6(CH_2CH=CH)_2(CH_2)_6-$	8,11-Icosadieno-[d]		Δ_2Ach
15	20:3(5,8,11)	$-(CH_2)_6(CH_2CH=CH)_3(CH_2)_3-$	5,8,11-Icosatrieno-[d]		Δ_3Ach
16	20:4(5,8,11,14)	$-(CH_2)_3(CH_2CH=CH)_4(CH_2)_3-$	5,8,11,14-Icosatetraeno-[d]	Arachidon-	Δ_4Ach
17	22:0	$-(CH_2)_{20}-$	Docosano-	Behen-	Beh
18	24:0	$-(CH_2)_{22}-$	Tetracosano-	Lignocer-	Lig
19	24:1	$-(CH_2)_7CH=CH(CH_2)_{13}-$	*cis*-15-Tetracoseno-	Nervon-	Ner
20	26:0	$-(CH_2)_{24}-$	Hexacosano-	Cerot-	Crt
21	28:0	$-(CH_2)_{26}-$	Octacosano-	Montan-	Mon

[a]Ending in "-ic" acid, "-ate," "-yl", for acid, salt or ester, acyl radical, respectively.

[b]Ending in "-ic acid," "-ate," "-oyl" for acid, salt or ester, or acyl radical, respectively.

[c]Not recommended because of confusion with caproic (hexanoic) and caprylic (octanoic) acids. Decanoic is preferred.

[d]Formerly "eicosa–" (Changed by IUPAC Commission on Nomenclature of Organic Chemistry, 1975.)

APPENDIX B

Symbols Recommended for Various Constituents of Lipids

Name	Symbol[a]
For alkyl radicals[b]	R
Methyl, Ethyl, . . .Dodecyl	Me, Et, Pr, Bu, Pe, Hx, Hp, Oc, Nn, Dec, Und, Dod
For aliphatic carboxylic acids[b]	acyl (not abbreviated), RCO-
Formyl, Acetyl, Glycoloyl, Propionyl,	Fo (or HCO), Ac, Gc, Pp,
Butyryl, Valeryl,	Bt, Vl,
Hexanoyl, Heptanoyl, Octanoyl,	Hxo, Hpo, Oco,
Nonanoyl, Decanoyl, Undecanoyl,	Nno, Dco, Udo,
Lauroyl, Myristoyl, Palmitoyl,	Lau, Myr, Pam,
Stearoyl, Eleostearoyl, Linoleoyl, Arachidonoyl	Ste, eSte, Lin, Δ_4Ach
For glycerol and its oxidation products[c]	
Glycerol, Glyceraldehyde, Glycerone, Glyceric acid	Gro, Gra, Grn, Gri.
For "glycosyl"	Ose
Glucose, Galactose, Fucose,. . .	Glc[d], Gal, Fuc. . .
Gluconic acid, Glucuronic acid	GlcA, GlcU[e]
Glucosamine[f], *N*-Acetylglucosamine	GlcN, GlcNAc
Neuraminic, Sialic, Muramic acids	Neu, Sia, Mur
N-Acetylneuraminic acid, *N*-Glycoloylneuraminic acid	NeuAc[g], NeuGc
Deoxy	d
Miscellaneous	
Ceramide, Choline, Ethanolamine,	Cer, Cho, Etn[h],
Inositol, Serine,	Ins, Ser,
Phosphatidyl, Sphingosine, Sphingoid, Phosphoric residue.	Ptd, Sph, Spd, *P*.

[a]These symbols are constructed in analogy to those already in use for amino acids and saccharides (11,13); they may assist the abbreviated representation of more complex lipids in a way similar to the peptides and polysaccharides. Prefixes such as "iso-," "tert-," "cyclo-" are specified in the symbols by lower-case superscripts (Pr^i, Bu^t, Hx^c) or lower-case prefixes (iPr, tBu, cHx), unsaturation by, e.g., Δ^3 for a 3,4 double bond, $\blacktriangle^3$ for a 3,4 triple bond (cf. Proteins, Vol. I, pp. 96-108, in *Handbook of Biochemistry*, 3rd edition, edited by G. Fasman, CRC Press, Cleveland, OH, 1976). Many of these symbols are drawn from previously published Recommendations (refs. 11,12). See also Appendix A.

[b]Systematic and recommended trivial names of unbranched, acyclic compounds only (cf. Appendix A). Other forms are created by prefixes (e.g., "iso-," "tert-," "cyclo-"). See Appendix A.

[c]These symbols form a self-consistent series for a group of closely related compounds. It is recognized that other abbreviations (but no symbols) are currently in use. (See Lip 2.12.)

[d]Not Glu (glutamic acid) or G (nonspecific).

[e]Recommended in place of GlcUA, the "A" being unnecessary.

[f]Approved trivial name for 2-amino-2-deoxyglucose; similarly for galactose (GalNAc), etc.

[g]AcNeu was recommended earlier (ref. 11). When it is necessary to differentiate between *N*-acetyl and *O*-acetyl derivatives, Neu*N*Ac and Neu*O*Ac (italicized locants, in contradistinction to GalNAc, etc.) may be employed.

[h]May take the form .OEtN < if substitution on the nitrogen atom is to be indicated.

APPENDIX C

Abbreviated Representation of Gangliosides

	Lipid Document[a]	Designation according to Wiegandt[b]	Svennerholm[c]
1.	I^3NeuAc-GalCer	G_{Gal} 1 NeuAc	–
2.	II^3NeuAc-LacCer	G_{Lac} 1 NeuAc	G_{M3}
3.	II^3NeuGc-LacCer	G_{Lac} 1 NeuNGl	–
4.	$II^3(NeuAc)_2$-LacCer	G_{Lac} 2 NeuAc	G_{D3}
5.	II^3NeuAc/NeuGc-LacCer	G_{Lac} 2 NeuAc/NeuNGl	–
6.	II^3NeuGc-LacCer	G_{Lac} 2 NeuNGl	–
7.	II^3NeuAc-$GgOse_3$ Cer	G_{Gtri} 1 NeuAc	G_{M2}
8.	II^3NeuAc-$GgOse_4$Cer	G_{Gtet} 1 NeuAc	G_{M1}
9.	IV^3NeuAc-n$LcOse_4$Cer	G_{Lntet} 1a NeuAc	$G_{M1\text{-}GlcNAc}$
10.	IV^6NeuAc-n$LcOse_4$Cer	G_{Lntet} 1b NeuAc	–
11.	IV^2Fuc,II^3NeuAc-$GgOse_4$Cer	G_{Gfpt} 1 NeuAc	–
12.	IV^3NeuAc-n$LcOse_4$Cer	–	–
13.	$II^3(NeuAc)_2$-$GgOse_4$Cer	G_{Gtet} 2b NeuAc	G_{D1b}
14.	IV^3NeuAc,II^3NeuAc-$GgOse_4$Cer	G_{Gtet} 2a NeuAc	G_{D1a}
15.	$II^3(NeuAc)_3$-$GgOse_4$Cer	G_{Gtet} 3b NeuAc	–
16.	IV^3NeuAc,$II^3(NeuAc)_2$-$GgOse_4$Cer	G_{Gtet} 3a NeuAc	G_{T1}
17.	IV^3NeuAc,$II^3(NeuAc)_3$-$GgOse_4$Cer	G_{Gtet} 4b NeuAc	–
18.	$IV^3(NeuAc)_2II^3(NeuAc)_3$-$GgOse_4$Cer	G_{Gtet} 5 NeuAc	–
19.	IV^3NeuAc,II^3NeuAc-$GgOse_5$Cer	G_{Gpt} 2a NeuAc	–

[a]To indicate linkage points and anomeric form: Fuc should be written (←1αFuc); NeuAc should be written (←2αNeuAc); $(NeuAc)_2$ should be written $(\leftarrow 2\alpha NeuAc8)_2$; etc. If these features are assumed or defined, the short form used in this column is more convenient for use in texts and tables.

[b]The subscripts to G (for ganglioside), from 7 on, have the meanings: Gtri =gangliotriose, Gtet = ganglio-tetraose, Litet = lactoisotetraose, Gpt = gangliopentaose, Gfpt = gangliofucopentaose [Ref. H. Wiegand, Hoppe-Seyler's Z. Physiol. Chem. 354:1049 (1973)].

[c]G = ganglioside, M = monosialo, D = disialo, T = trisialo. Arabic numerals indicate sequence of migration in thin-layer chromatograms [Ref. Svennerholm, L., J. Neurochem. 10:613 (1963)].

Interrelationship between the Dietary Regulation of Fatty Acid Synthesis and the Fatty Acyl-CoA Desaturases

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ABSTRACT

In this paper we present further evidence for the close control of fatty acid synthetase and stearoyl-CoA desaturase. Furthermore, we have established that whereas dietary palmitic acid may influence the activity of this desaturase but not of fatty acid synthetase, dietary linoleic acid appears to control both these enzymes. Finally, we have studied the influence of dietary fat and carbohydrate on the activities of the Δ^6 and Δ^5 desaturases. The former is only slightly affected by these dietary components. The Δ^5 desaturase activity is stimulated as the dietary fat content rises but is unaffected by dietary carbohydrate. The control of these enzymes is therefore independent of the control of fatty acid synthetase and stearoyl-CoA desaturase. From the data presented, the magnitude of the controlling effect of polyunsaturated fatty acids on fatty acid synthetase and stearoyl-CoA desaturase activity is determined and its relevance to lipogenesis in man based on daily intake of carbohydrate and linoleic acid is discussed.

INTRODUCTION

A recent review by Volpe and Vagelos (1) discusses at length the regulation of the biosynthesis of saturated fatty acids via acetyl-CoA carboxylase and fatty acid synthetase. It is these enzymes which provide, in part, the endogenous substrates for the microsomal Δ^9 desaturase. Although it has been known for many years that dietary carbohydrate can result in the induction of stearoyl-CoA desaturase (2) as well as several other lipogenic enzymes (3), little is known about the detailed control of desaturases. In general terms, the control of fatty acid biosynthesis appears to be at two levels. First is the metabolic or allosteric control of acetyl-CoA carboxylase. This is manifested by the competition between citrate and palmitate for binding sites on the enzyme protomers, which results in activation (association) and deactivation (dissociation) of the enzyme, respectively. Second is the control at the level of enzyme synthesis which could be considered as a long term control mechanism. Similarities in the behavior of acetyl-CoA carboxylase, fatty acid synthetase, and stearoyl-CoA desaturase to changes in the diet suggest that these may be linked as a unit by a common control mechanism. However, detailed studies have not yet been carried out, and this paper describes work on the direct comparison of two of these enzymes as influenced by dietary carbohydrate and lipid.

MATERIALS AND METHODS

Diets

All feeding experiments were carried out with groups of six male litter weanling rats of the Colworth-Wistar strain. Animals were fed ad libitum for 14 days on either a control diet consisting of 64% starch, 25% casein, 4% minerals, 6% cellulose powder, 0.15% cystine, and vitamins 0.53% or a test diet in which some of the starch had been replaced by other carbohydrates or lipid.

In the preliminary studies to examine the effect of different lipid types on enzyme activities, dietary starch was replaced by an equal weight of the lipid under investigation. Consequently, the calorie content of the control starch based diet was lower than the lipid supplemented diets, which were all comparable. In subsequent studies designed to investigate the specific effect of linoleic and palmitic acids on the control of enzyme activities, all diets were essentially isocaloric.

The total amount of fat ingested was determined from the food intakes and the percentage fat added to the diet.

Enzyme Assays

Fatty acid synthetase was assayed according to the method described by Bruckdorfer et al. (4) and the Δ^9 desaturase as described by Jeffcoat et al. (5). The Δ^6 and Δ^5 desaturases were assayed for 10 min at 37 C using 2.5 mg and 1 mg of microsomal protein, respectively. The incubations in a total volume of 2.5 ml of 0.1 M potassium phosphate buffer pH 7.4, contained 20 nmoles of either [1-^{14}C]linoleic acid (The Radiochemical Centre, Amersham, England) or [1-^{14}C]eicosatrienoic acid (New

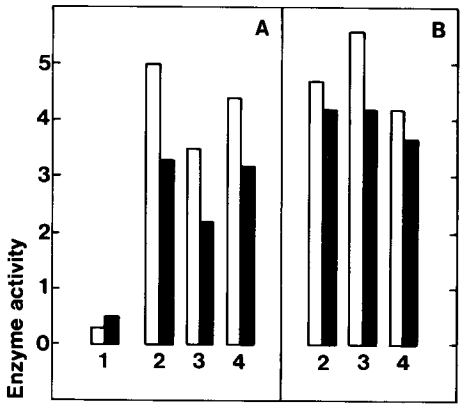

FIG. 1. The effect of dietary carbohydrates on the activity of fatty acid synthetase and stearoyl-CoA desaturase. In the continuous feeding (A) and starving/refeeding (B) situation fatty acid synthetase activity is expressed as μmoles NADPH oxidized per min per g liver (□) and stearoyl-CoA desaturase as nmoles oleate produced per min per mg microsomal protein (■). 1. Purified diet, 2. 74% (w/w) sucrose, 3. 74% (w/w) glucose, 4. 74% (w/w) fructose.

England Nuclear, Winchester, England). The following concentrations of cofactors were also used: 9 μmoles ATP, 10 μmoles $MgCl_2$, 700 nmoles NADH, 360 nmoles NADPH, and 130 nmoles coenzyme A. The percentage desaturation was determined by gas liquid chromatography of the methyl esters of the fatty acids (5).

All enzyme assays were carried out using either the 100,000 x g supernatant or the microsomal fraction from six livers from rats on each of the various diets. Each incubation was carried out with two or three concentrations of protein to ensure the linear enzyme dependence of the reaction.

RESULTS AND DISCUSSION

It is now generally accepted that in mammalian systems a high carbohydrate/low fat diet results in enhanced activity of acetyl-CoA carboxylase (1), fatty acid synthetase (1), elongase (6), stearoyl-CoA desaturase (2), and the biosynthesis of triacylglycerol (7,8). However, little is known about the detailed control mechanisms which enhance enzyme activity or result in the biosynthesis of new enzyme protein. It has been suggested that high carbohydrate diets could result in elevated levels of citrate which is known to activate acetyl-CoA carboxylase (9). This enzyme is thought by some to be the rate-limiting step in the biosynthesis of saturated fatty acids (9) and thus an increase in this activity would result in an overall increase in fatty acid synthesis. This in turn might result in enhanced stearoyl-CoA desaturase activity via adaptive enzyme formation and indeed there is some evidence to support this hypothesis. Mercuri et al. (10) have shown that in the case of streptozotocin induced diabetic rats the decreased level of the Δ^9 desaturase can be elevated by feeding fructose, glycerol, or saturated fatty acids. This is in contrast to our own observations reported here where we have attempted to understand the dietary factors which influence the activities of the fatty acid synthetase, and the Δ^9, Δ^6, and Δ^5 desaturases.

The Influence of Carbohydrate Diets on Δ^9 Desaturase

Bruckdorfer et al. (4) demonstrated that feeding rats a high carbohydrate diet resulted in enhanced levels of fatty acid synthetase. In particular, they showed that of the sugars tested (maltose, glucose, sucrose, starch, and fructose) fructose gave the highest levels of fatty acid synthetase activity in the liver. Although Oshino and Sato (2) had demonstrated that diets rich in sucrose were also effective in raising the levels of Δ^9 desaturase, no work has been reported on the effect of various dietary sugars on the Δ^9 desaturase. We therefore investigated the effect of feeding rats diets containing 15% casein, 4% minerals, 5% vitamin mix, 2% cellulose powder, and either 74% sucrose, glucose, or fructose. The results from a 14 day continuous feeding study are shown in Figure 1A and from a 24 hr starvation followed by a 17 hr refeeding period after 14 days continuous feeding are shown in Figure 1B. The close correlation between fatty acid synthetase and Δ^9 desaturase activity would support the hypothesis that the latter was induced by adaptive enzyme formation. To investigate this in more detail, the effect of saturated as well as unsaturated dietary fatty acids on the level of activity of fatty acid synthetase Δ^9, Δ^6, and Δ^5 desaturase was studied.

The Influence of Dietary Fats on Desaturase Activity

Rats were fed ad libitum for fourteen days either a control diet or diets in which 20% of the diet as starch had been replaced by an equal weight of various lipids or sucrose. The specific activities of the various enzymes relative to their value on the purified diet are given in Table I. The most pronounced effects are seen with the high carbohydrate/low fat diets which elevate the levels of fatty acid synthetase and the Δ^9 desaturase but have comparatively little effect on the activities of the Δ^6 and the Δ^5 desaturases. Conversely, a high fat diet seems to stimulate the activity of the Δ^5 desaturase and

TABLE I

The Influence of Dietary Fat on the Levels of Hepatic Fatty Acid Synthetase, Δ9, Δ6, and Δ5 Desaturase

Diet	FAS[a]	Δ9[b]	Δ6[c]	Δ5[d]
Control	1.00	1.00	1.00	1.00
Sucrose	2.29	1.54	1.25	1.08
Tallow	0.34	0.41	0.99	1.18
Hydrogenated tallow	0.46	0.81	1.07	1.58
Corn oil	0.19	0.25	0.75	1.72
Triolein	0.40	0.56	0.88	2.45
Spital	0.34	0.30	0.59	-

[a]Fatty acid synthetase 1.35 μmoles NADPH oxidized per min per g liver.

[b]Stearoyl-CoA desaturase 1.33 nmoles oleate produced per min per mg protein.

[c]Linoleoyl CoA desaturase 284 μmoles γ-linolenate produced per min per mg protein.

[d]Eicosatrienoic acid desaturase. 893 pmoles arachidonate produced per min per mg protein.

TABLE II

Dietary Intake Expressed as g Fatty Acid Ingested per Rat Over 14 Days

Diet	Fatty acid				
	16:0	18:0	18:1	18:2	Total
Control	0.17	0.06	0.15	0.10	0.60
Sucrose	0.12	0.04	0.12	0.05	0.40
Tallow	8.70	6.91	13.75	1.15	33.9
Hydrogenated tallow	11.86	24.98	-	-	40.6
Corn oil	3.56	0.51	8.45	18.68	31.8
Triolein	0.48	0.48	19.46	-	21.0
Spital	2.09	0.24	2.0	2.7	8.0

inhibit the activity of the fatty acid synthetase and the Δ^9 desaturase. It is perhaps not surprising that dietary fats limit the activity of the fatty acid synthetase, but an obvious explanation for the apparent stimulation of the Δ^5 desaturase activity is less clear. However, from the known inhibitory effect of palmitic acid and palmitoyl-CoA on the fatty acid synthetase and acetyl-CoA carboxylase, it might be predicted that diets with high levels of this fatty acid would be the most effective in controlling the activities of these two enzymes. From Table II, it is apparent that this is not the case and in fact those rats fed a corn oil supplemented diet showed the lowest activity. Furthermore, the rats fed a Spital diet (a commercial diet composed of high carbohydrate and low fat) showed considerably depressed levels of fatty acid synthetase unlike those animals fed a sucrose supplemented diet. Since the high carbohydrate effect was being compensated by some other component, attention was focused upon the fatty acid intake. Considering that rats on a corn oil supplemented diet showed the lowest fatty acid synthetase activity, it seemed likely that the relatively high linoleic acid intake might be playing a contributory role.

The Influence of Dietary Ethyl Linoleate on Fatty Acid Synthetase and Δ^9 Desaturase

Six male litter weanlings were allocated to four dietary groups. Group 1 was fed ad libitum a 20% (w/w) supplemented diet and Groups 2-4 20% (w/w) sucrose with 0.5, 1.0 and 1.5% (w/w) ethyl linoleate, respectively. At the end of 14 days the rats were killed, their livers removed, homogenized and fractionated to allow measurements of the fatty acid synthetase and Δ^9 desaturase to be made. The results are shown in Figure 2. A control experiment was also set up in which the ethyl linoleate was replaced by ethyl palmitate.

In both series of experiments, a fifth group were fed on Spital. From these results, it was apparent that dietary ethyl linoleate could influence the activity of both fatty acid synthetase and the Δ^9 desaturase. Ethyl palmitate over a similar concentration range had no effect at all on the fatty acid synthetase but partially inhibited the Δ^9 desaturase activity.

These results are in agreement with the view that short term control of fatty acid synthetase is at the level of acetyl carboxylase manifested via the dissociation/association of the enzyme protomers whereas the long term control is at

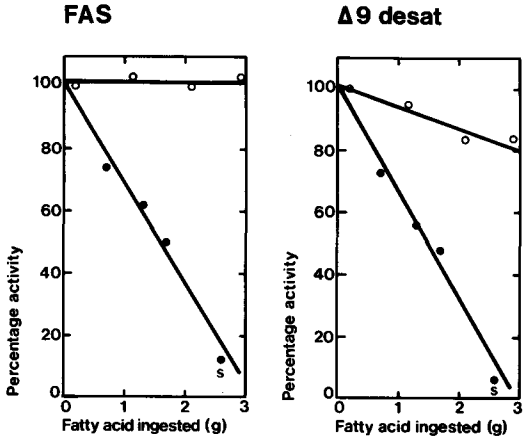

FIG. 2. The effect of dietary fatty acid ethyl esters on the activity of fatty acid synthetase and stearoyl-CoA desaturase. Data are expressed as percentage activity, relative to the enzyme activity determined from rats on a fat-free diet, as a function of the total amount of the specific fatty acid ingested per rat during the fourteen day feeding trial. The open symbols (○) refer to the enzyme activities when the diets were supplemented with ethyl palmitate and the closed symbols (●) ethyl linoleate. The 100% value is obtained from animals fed a diet consisting of 20% (w/w) sucrose at the expense of the starch. The closed symbols corresponding to 2.7 g of ingested ethyl linoleate (s) represent the value of ingested palmitic and linoleic acids from the Spital diet.

the level of the fatty acid synthetase. The former was brought about by competition between palmitic acid and citric acid and the latter by induced enzyme synthesis possibly under hormonal control. The Δ^9 desaturase is thought to be induced via adaptive enzyme formation independently of hormonal control by the increased flux of saturated fatty acids produced by the enhanced activity of fatty acid synthetase. These observations have come from the work of Mercuri et al. (10) who showed that in the normal or diabetic rat the level of Δ^9 desaturase activity could be raised by feeding saturated fatty acids. Our results reported here suggest that palmitic acid, while not affecting fatty acid synthetase activity, actually diminished the activity of the Δ^9 desaturase. However, it was only about one-fifth as effective as linoleic acid, and it is clear from the data shown in Figure 2 that it is not the palmitic acid content of the Spital diet which is contributing to the depressed level of the enzyme activities. Extrapolation of the experimental data would indicate that linoleic acid is the major contributing fatty acid.

A recent report by Huntley (11) refers to the close correlation between the activities of acetyl-CoA carboxylase, fatty acid synthetase, and the Δ^9 desaturase in maternal, foetal, and neonatal rats. Furthermore, Huntley also refers to the linoleic acid sensitivity of the desaturase, an observation which has been referred to by Muto and Gibson (12) and tentatively by Inkpen et al. (13). In these last experiments, the level of Δ^9 desaturase was compared in groups of rats fed either stimulatory amounts of hydrogenated coconut oil or diets in which this fat had been replaced by 5% increments of safflower oil. The authors conclude that the decrease in desaturase activity is a function of the increasing linoleic acid content of the diet due to the safflower oil although the results could equally well be explained in terms of decreasing amounts of coconut oil. Our data based on feeding experiments utilizing carbohydrate fat free diets supplemented with increasing amounts of ethyl linoleate enable us

TABLE III

Food Intakes and Body Weights of Rats Fed a High Carbohydrate Diet Supplemented with Either (A) Ethyl Palmitate or (B) Ethyl Linoleate[a]

	Control	Spital	Fatty acid supplemented diet		
(A) Ethyl Palmitate					
Food intake (g)	238	282	232	226	217
Initial body wt. (g)	59	58	58	60	59
Final body wt. (g)	135	141	138	140	134
Increase in body wt.	2.3	2.4	2.4	2.3	2.3
(B) Ethyl Linoleate					
Food intake (g)	176	206	170	173	153
Initial body wt. (g)	42	42	41	41	40
Final body wt. (g)	106	117	102	104	100
Increase in body wt.	2.5	2.8	2.5	2.5	2.5

[a]All values represent an average of six determinations—one from each of the six animals in each dietary group. Food intakes are expressed as the average value of food ingested per rat over the fourteen day feeding trial. The three sets of data for fatty acid supplemented diets represent diets supplemented with ca. 0.5, 1.0, and 1.5% fatty acid ethyl ester.

to quantitate the magnitude of the effect of this fatty acid on the fatty acid synthetase and Δ^9 desaturase. It can be calculated from the known food intakes, Table III, and the percentage of fatty acid composition of the diet, that for every 3 g of linoleic acid ingested, each rat also ingest 54 g of sucrose during the 14 day feeding period. From these observations, it can be shown that linoleic acid on a weight for weight, calorie for calorie, or mole for mole basis is 18, 8, and 15 times more effective, respectively, in repressing these enzymes than sucrose is in inducing them.

The nature of the linoleic acid control is not yet understood and so a possible link between the inhibitory effect of this essential fatty acid and the stimulatory effect of carbohydrate was sought. Mercuri et al. (10) have shown that in the diabetic rate 75% of the normal stearoyl-CoA desaturase activity can be restored by feeding fructose which is known to be metabolized by insulin independent reactions in the liver (14,15). It has also been demonstrated that during fasting the blood insulin levels decrease as does the liver fatty acid synthetase and the Δ^9 desaturase activities. After feeding, the levels of the enzymes and the hormone rise in parallel. If the inhibition of the lipogenic enzymes by dietary linoleic acid is a reflection of the insulin production, then elevated levels of fatty acid synthetase and the Δ^9 desaturase brought about by dietary fructose should not be repressed by dietary linoleic acid. In order to test this possibility, four groups of rats were fed either 20% glucose, 20% glucose and 3% corn oil, 20% fructose, or 20% fructose and 3% corn oil. The results are shown in Table IV. They show that dietary corn oil (60% linoleic acid) is less inhibitory when fructose rather than glucose is used to induce these two enzymes. However, the data do suggest that the major inhibitory effect of the linoleic acid cannot be explained by a mechanism which involves the control of insulin.

Finally, it is worth considering the quantitative controlling effect of the dietary linoleic acid and its possible relevance to the human situation. From Figure 2 it has already been calculated that dietary linoleic acid has a sensitive control over certain lipogenic enzymes. On a weight for weight basis, it can be shown that 2-2.5 g linoleic acid ingested per day per kilo body weight can completely suppress the inductive effect of 20% dietary sucrose on the hepatic fatty acid synthetase and Δ^9 desaturase activity in the rat. Extrapolation of these values would mean that the daily intake of a 75 kilogram man would have to be 150-190 g linoleic acid per day. This is clearly meaningless since

TABLE IV

The Inhibitory Effect of Dietary Corn Oil on the Induction of Fatty Acid Synthetase and Δ9 Desaturase by Glucose and Fructose

Diet	FAS[a]	Δ9 Desaturase[b]
20% glucose	2.1	2.9
20% glucose 3% corn oil	0.8	1.5
20% fructose	2.2	4.6
20% fructose 3% corn oil	1.2	3.2

[a]Fatty acid synthetase is expressed as μmoles NADPH oxidized per min per g liver.

[b]Δ9 Desaturase is expressed as nmoles oleate produced per min per mg microsomal protein.

average U.K. food intakes calculated from the Household Food Composition and Expenditure, HMSO, 1971, show that the actual value is only 10-15 g of linoleic acid per day. The daily carbohydrate intake calculated from the same source indicated ca. 30% of the 300 g of ingested carbohydrate is mono- and disaccharide. From Figure 2 it has been calculated that 1 g of linoleic acid can completely suppress the inductive effect of 18 g of sucrose. The 10-15 g of linoleic acid ingested daily by man should, on this basis, assuming a similar metabolism and control as in the rat, be capable of suppressing the inductive effect of 180-270 g of dietary sucrose. From the data quoted for the dietary intake of mono- and disaccharides (ca. 100-200 g per day), it becomes apparent that the daily intake of essential fatty acid should be adequate to prevent over-synthesis of fatty acid and synthetase and Δ^9 desaturase.

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Analysis of Oleate and Linoleate Hydroperoxides in Oxidized Ester Mixtures[1]

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ABSTRACT

The hydroperoxides in oxidized mixtures of methyl oleate and linoleate were reduced to the corresponding hydroxyesters, which were separated from unoxidized esters by thin layer chromatography on silica gel. The hydroxyesters from oleate and linoleate were converted to trimethylsilyl ethers and separated by gas chromatography on OV 225. The results suggest that methyl linoleate oxidizes about ten times faster than methyl oleate, but oleate hydroperoxides are formed in appreciable amounts, even in mixtures containing 87% methyl linoleate.

INTRODUCTION

Peroxide values often are used to measure the extent of oxidation of lipids, but peroxide values give a composite measure when more than one fatty acid is present so that one does not know how much each of the fatty acids in the mixture has oxidized. Comparisons have been made of the rates of oxidation of purified oleate, linoleate, and linolenate esters (1,2). These comparisons showed that linoleate oxidized about 10.5 times faster than oleate, and linolenate two times faster than linoleate. Rates obtained in this way do not distinguish the initiation and propagation rates; thus, they do not reveal what the relative rates of oxidation of the individual fatty acids might be in a mixture with the same initiation rate. The relative rates of oxidation under such circumstances can be obtained from a comparison of the propagation rate constants, k_p, which cannot be measured directly. By the rotating sector technique, Bateman et al. (3) measured a combination of the propagation and termination rate constants, $k_p/k_t^{1/2}$, at 65 C and found the values for linoleate to be about ten times greater than for oleate. By a similar technique, Howard and Ingold (4) found $k_p/k_t^{1/2}$ in a ratio of 24:1 for linoleate and oleate at 37 C. By a separate measure of k_t, they found k_p's in a ratio of 70:1 for linoleate-oleate.

The addition of small amounts of linoleate to oleate greatly increased its rate of oxidation (1,5). Recently Rosas Romero and Morton (6) examined the rate of oxidation of oleate-linoleate mixtures and found that the rate constant for the mixture was a linear function of the oleate concentration at low peroxide values, but, when the peroxide values were large enough that the initiation rate became bimolecular, the rates were a complex function of the oleate concentration. They attributed this nonlinearity to differences in the propagation and termination rates of the two fatty acids.

Previously, we have tried to measure the ratio of methyl oleate and linoleate hydroperoxide in oxidized mixtures by reduction of the peroxide to the corresponding hydroxyester, acylation of the alcohol function, and fractionation of the linear unoxidized esters from the branched acylated esters with urea. The acyl groups were removed, and the hydroxyesters were separated and estimated by densitometry on thin layer chomotograms (TLC) (7,8). The separation of the hydroxyesters of acylated hydroxyesters derived from oleate and linoleate by TLC has proved difficult to reproduce. In this paper, we wish to report our efforts to achieve this analysis by gas liquid chromatography (GLC) of the trimethylsilyl ethers (TMS-ethers).

METHODS

Methyl oleate and methyl linoleate were prepared by the urea fractionation of olive and safflower oils, respectively. Methyl ricinoleate was prepared by chromatography of castor oil methyl esters on silica gel. Methyl 12-hydroxystearate was prepared by hydrogenation of methyl ricinoleate with a palladium catalyst. Methyl oleate and linoleate mixtures were prepared immediately after distillation of the purified esters. Impurities that interfered with subsequent analysis were sometimes found in the distilled esters. These were removed by passing the esters through an alumina column. The ratios of oleate to linoleate in mixtures were determined by gas chromatography on EGSS-X (Applied Science Laboratories, State College, PA).

About 10 g of each methyl ester mixture was oxidized at 28 C. Peroxide values were

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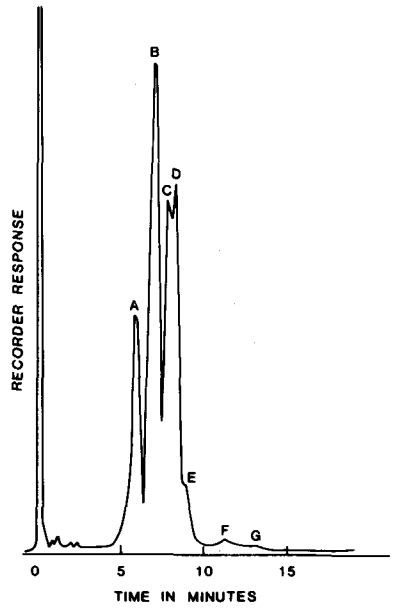

FIG. 1. Typical gas liquid chromatography (GLC) of trimethylsilyl (TMS)-ester mixture. A: TMS-oleate; B-E: TMS-linoleate; F, G: decomposition products.

determined periodically by the Stamm method (9). When the peroxide value reached ca. 5, 10, 20, and 40, samples were withdrawn, and the peroxides were reduced to alcohols with iodide (10). The esters were extracted into chloroform, washed with water and 5% sodium bicarbonate solution and dried with sodium sulfate. The solvent was removed in a rotary evaporator at reduced pressure while the temperature was kept below 50 C.

To separate the methyl hydroxyesters from the unoxidized fatty acids, TLC on Silica Gel G was used. The plates (0.25- or 0.75-mm thick) were activated at 100 C for 1 hr and stored in a dry chamber. The sample (150 mg) was applied to the plate with a streaker (Applied Science Laboratories). Guide spots of methyl ricinoleate were applied to each side of the plate, and the plate was developed in hexane-diethyl ether (70:30, v/v). The bands were located by spraying with 2',7'-dichlorofluorescein and viewing under ultraviolet light. The methyl hydroxyester band was extracted from the plate with 15 ml of ether. The methyl ricinoleate guide spots were used to indicate the area to be extracted when the streak was not visible.

The methyl hydroxyesters were converted to TMS-ethers as follows. The sample was dissolved in 1 ml of pyridine and 2 ml of hexane, and 0.2 ml of hexamethyldisilazane and 0.1 ml trimethylchlorosilane were added with rapid mixing for 15-30 sec. After 4 hr, 5 ml of hexane and 5 ml of water were added, and the hexane layer was recovered. The water layer was washed twice more with hexane, and the combined hexane extracts were dried with sodium sulfate and evaporated in a stream of nitrogen.

Methyl heptadecanoate was added to the TMS-ethers as an internal standard, and the mixture was analyzed by GLC at 205 C on a 180 cm x 0.32 cm stainless steel column packed with 10% OV 225 on 80/100 mesh Chromosorb W(HP).

Mass spectra were obtained on a DuPont 21490 gas chromatograph-mass spectrophotometer. Infrared spectra were obtained with a Beckman IR 12 spectrophotometer; visible and ultraviolet spectra, with a Beckman DU spectrophotometer.

cis-trans Isomerization was achieved according to Nichols et al. (11) by exposure to iodine in hexane for 4 hr.

RESULTS AND DISCUSSION

Silylation Conditions

Conditions for the optimum yield of silyl ether with the methyl hydroxyesters were studied with methyl ricinoleate and hydroxyesters isolated from oxidized methyl oleate and linoleate. Yields were increased by ca. 7% by using a 2:1 ratio of hexane to pyridine as reaction solvent, probably because the hexane suppresses side reactions that depend on ionic mechanisms. Optimum yields for methyl ricinoleate were obtained at 45 min. Longer times were required for the maximum yield with hydroxyesters isolated from methyl oleate and linoleate, which required 2 and 4 hr, respectively. Evidently, double bonds adjacent to the hydroxy group slow the reaction. Removal of the solvents and reagents from the reaction mixture in a stream of nitrogen, as commonly practiced, led to variable results. Extraction with hexane after the addition of water was more reproducible. Yields were not improved at temperatures higher or lower than room temperature (about 25 C). At 70 C, yields began to decrease. GLC of the silyl derivatives of the unsaturated esters changed on storage; therefore, quantitative methods should be completed within less than 2 days after the reaction, and during this time, the derivatives should be stored in solvent in a freezer and protected from oxidation.

Properties of the TMS-Ethers

The TMS-ether produced from the hydroxyester isolated from oxidized methyl oleate was homogeneous by TLC and GLC. The infrared

spectrum indicated a TMS group (845, 1250 cm^{-1}), a *trans* double bond (970 cm^{-1}), and ester (1173, 1745 cm^{-1}).

The TMS ether produced from the hydroxyester isolated from oxidized methyl linoleate yielded four poorly resolved peaks on GLC (Fig. 1). Upon hydrogenation, the four peaks merged into one, with a retention time corresponding to the TMS-ether of methyl 12-hydroxystearate and a mass spectrum with major peaks of masses 173, 229, 259, and 315, corresponding to fragmentation of the TMS-ether of methyl 9-hydroxystearate and methyl 13-hydroxystearate. On TLC, the TMS-ether yielded one main spot, with a light spot moving at slower speeds. Isolation and GLC of the lower spot gave peaks that corresponded to F and G in Figure 1. F and G are degradation products, and if the TMS-ether is stored, they become larger while peaks A through E decrease. The ultraviolet spectrum of the band corresponding to peaks B through E had a peak at 233 nm, indicating diene conjugation. The band corresponding to peaks F and G had a broad absorption maximum at 278 nm, suggesting conjugated triene or ketodiene. The infrared spectrum of the TMS-ether indicated TMS groups (845, 1250 cm^{-1}), ester (1173 and 1745 cm^{-1}) and *cis-trans* conjugation (950, 982 cm^{-1}). The four peaks are believed to be caused by *cis-trans* and positional isomers. When the TMS-ether was treated with iodine, the ultraviolet absorption maximum shifted from 233 to 231 nm as found by Nichols et al. (11), the infrared peak at 950 cm^{-1} disappeared and the peak 982 cm^{-1} shifted to 988 cm^{-1}, typical of a shift to *trans-trans* conjugation (12,13). GLC revealed that iodine treatment caused a marked decrease in the first of the four peaks and an increase in the last.

GLC Response

The silylation procedure seemed to give complete reaction with very little side reaction, but the GLC response of the TMS derivatives was low compared with that of the methyl heptadecanoate standard. The responses of the TMS-ethers of methyl ricinoleate and methyl 12-hydroxystearate were both 78% of the value of an equimolar amount of methyl heptadecanoate. The response of the TMS derivatives of the hydroxyesters produced from the oxidation of methyl oleate and methyl linoleate was determined as follows. Methyl oleate and methyl linoleate were each oxidized to peroxide values of ca. 5, 10, 20, and 40. Aliquots were carried through the procedure, and the response factors were compared with that of a methyl heptadecanoate internal standard. The

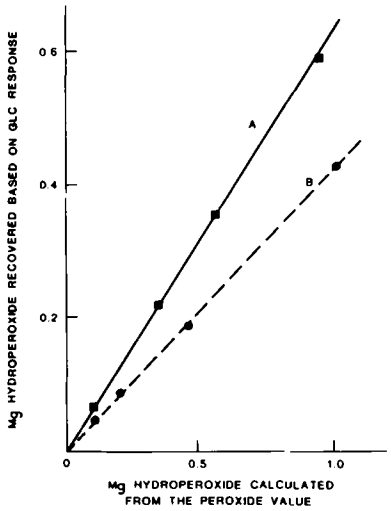

FIG. 2. Gas liquid chromatographic (GLC) response of trimethylsilyl (TMS)-oleate (A) and TMS-linoleate (B) based on a methyl heptadecanoate internal standard vs. the weight of peroxide calculated from the peroxide value.

amount of hydroperoxide indicated by GLC was compared with that expected from the peroxide values. The results are shown in Figure 2. From the slopes of the lines, one can calculate responses of 63% of that of an equivalent weight of methyl heptadecanoate for methyl oleate and 40% for methyl linoleate. It is not clear whether the responses are low entirely because of the flame detector or because of losses in reactions and separations. In any event, Figure 2 and the recoveries reported below for mixtures of methyl oleate and linoleate show that the results are reproducible.

Analysis of Oxidized Ester Mixtures

Mixtures of methyl oleate and linoleate were prepared and oxidized to various peroxide values. The amounts of the TMS-ethers produced were measured by using the appropriate GLC response factor. Results are shown in Table I. Recoveries ranged from 86 to 105% of the value expected from the peroxide determination. The percentage of linoleate hydroperoxide in the total hydroperoxides was approximately constant as the peroxide value increased. The four GLC peaks representing the TMS product of linoleate (B through E in Figure 1) showed no change in ratio with the extent of oxidation or with the composition of the ester mixture being oxidized.

According to Russell (14) two co-oxidizing substrates should follow the equation:

$$\frac{d(R^1H)}{d(R^2H)} = \frac{d(R^1OOH)}{d(R^2OOH)} = \frac{1 + r_1 (R^1H/R^2H)}{1 + r_2 (R^2H/R^1H)} \quad (I)$$

TABLE I

Analysis of the Hydroperoxides Formed in a Mixture of Methyl Oleate and Methyl Linoleate

% Methyl linoleate	PV[a]	% Recovery[b]	% Linoleate hydroperoxide
87.4	7.5	91.6	94.4
	28.1	95.0	94.7
	40.6	88.4	90.1
73.4	7.2	94.6	91.9
	16.0	86.0	93.6
	42.2	86.2	94.3
62.3	5.2	105.1	91.2
	10.5	104.0	91.2
	25.9	106.3	90.5
	53.9	103.0	91.3
32.0	5.4	98.8	88.4
	13.2	91.7	82.8
	24.3	90.7	87.9
	46.9	86.3	83.9
25.2	6.5	89.4	71.2
	10.6	97.2	71.7
	18.2	105.0	71.6
	41.9	87.5	71.9

[a]Peroxide value.

[b]Gas liquid chromatography yield compared with peroxide values.

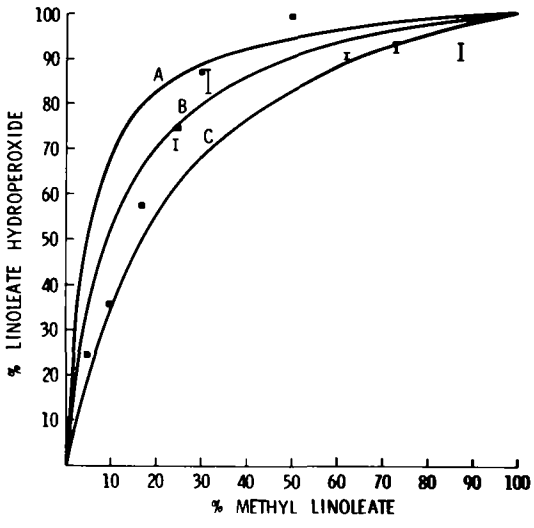

FIG. 3. Percentage of linoleate hydroperoxide in the total hydroperoxides vs. the percentage of methyl linoleate in an oleate-linoleate mixture. The curves are based on Russell's equation. Curve A assumes r_1 20 and r_2 0.05, curve B assumes r_1 10 and r_2 0.1, curve C assumes r_1 5 and r_2 0.2. The bars show the range of the present data. The points are derived from the data of Yanishlieva and Popov.

where RH is the substrate, ROOH the hydroperoxide, superscripts 1 refer to linoleate and superscripts 2 refer to oleate, r_1 is the ratio of the propagation constants k^{11}/k^{12}, and r_2 is k^{22}/k^{21}; k^{11} would be the constant for the attack of linoleate peroxy radical on linoleate; k^{12} would be the constant for the attack of linoleate peroxy radical on oleate, etc. Theoretically, $r_1 \cdot r_2$ should equal approximately 1. Ross (15) showed that this equation can be rearranged to give a linear plot with a slopt of $-r_2$ and an intercept of r_1, but the data in Table I do not give a stright line.

A plot of the data of Table I and of Russell's equation, assuming that r_1 is 5, 10 and 20 and r_2 is 0.2, 0.1 and 0.05 is given in Figure 3. These values of r_1 and r_2 assume that linoleate is attacked about 5, 10, or 20 times faster than oleate, which is lower than rate constant determinations would predict (4), but higher values of r_1 give even poorer fits of our data. The persistence, even at high percentages of methyl linoleate, of substantial proportions of oleate hydroperoxide in the mixtures that we analyzed is particularly interesting.

Another way to determine the proportion of linoleate and oleate hydroperoxides in a mixture is to measure the peroxide value and the diene conjugation. The latter should be a measure of the linoleate hydroperoxide, and the oleate hydroperoxide can be calculated by the difference between the total and linoleate hydroperoxide. To do this, one needs to know the ultraviolet absorption constant for the linoleate hydroperoxide, and this is affected by *cis-trans* isomerism (13) and the oxygen concentration (16). Data reported by Yanishlieva and Popov (17) were used to make such a calculation and are also included in Figure 3. The data of Yanishlieva and Popov also fail to give a straight line in a Ross plot, but fit the theoretical curve about as well as our data. In Yanishlieva and

Popov's data the percentage of linoleate hydroperoxide rises steeply and reaches 100% at only 50% linoleate, in contrast to our data.

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Studies on the Inhibition of the Desaturases by Cyclopropenoid Fatty Acids[1]

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ABSTRACT

Unwashed rat liver microsomes were used to study the inhibition of the Δ^6 and Δ^9 desaturases by cyclopropenoid fatty acids with the ring structure about the 9,10 or 6,7 carbon atoms. The 9,10 cyclopropenoid acid (sterculic acid) is shown to be an effective inhibitor of only Δ^9 desaturase and then only in the presence of $MgCl_2$ and coenzyme A (presumably due to the formation of sterculoyl-CoA). Two 6,7 cyclopropenoid acids of different chain lengths showed no marked inhibition of either the Δ^6 or Δ^9 desaturase. By the use of [^{3}H]-sterculic acid, it has been shown that under conditions of high inhibition of the Δ^9 desaturase the inhibitor is not covalently attached to the enzyme at any point. This disproves older ideas on the mechanism of inhibition that assumed reaction between the cyclopropenoid ring and sulphydryl groups on the enzymes.

INTRODUCTION

When sterculic acid [8-(2-octyl-1-cyclopropenyl) octanoic acid] is included in the diet of hens (1), pigs (2), and cows (3), there is an accumulation of stearic acid and an apparent loss of oleic acid both in tissues and in the lipids of egg yolk and milk. This compositional change is due to a marked inhibition of the Δ^9 desaturase of the tissues that converts stearic acid to oleic acid and utilizes the acyl-CoA thiolesters as substrate. Inhibition of this enzyme also occurs with the rat (4) and the single-celled alga, *Chlorella vulgaris* (5). There is one singular feature of the inhibition that has never been satisfactorily explained and that is that though sterculic acid inhibits the conversion of stearic to oleic acid where the starting material is labeled stearic acid, no inhibition is observed when labeled acetate is the precursor (4,5). This, together with the observation that high concentrations of many fatty acids show some inhibition of the Δ^9 desaturase, has led to three theories of cyclopropenoid inhibition:

(a) The effect is nonspecific and is due to a detergent-like effect on the enzyme (6).
(b) The effect is not specific for the desaturase enzyme that converts the form of "activated" stearic acid that the tissue forms from added stearic acid, but the effect takes place at some transfer stage of the activated stearic acid to the enzyme (5).
(c) There are two mechanisms for the formation of oleic acid, one involving the direct desaturation of stearoyl-CoA that is inhibited by sterculic acid and the other an undefined mechanism that produces oleic acid by chain elongation of some shorter chain unsaturated acid (4).

The outstanding questions are therefore (a) in what form does sterculic acid act as inhibitor, (b) is this inhibition demonstrated at lower concentrations than other fatty acids, (c) does the cyclopropenoid group have to be at the same position in the chain as the double bond to be introduced into the true substrate, (d) is the Δ^6 desaturase that normally converts linoleic acid to γ-linolenic acid inhibited by 6,7 or 9,10 cyclopropenoid acids, and (e) is the inhibitory cyclopropenoid fatty acid covalently bonded to the desaturase enzyme?

It is the purpose of this paper to try to answer these questions.

MATERIALS AND METHODS

Substrates and Inhibitors

1-[^{14}C]-palmitic, stearic, linoleic, and α-linolenic acids were obtained from the Radiochemical Centre, Amersham, Bucks. 1-[^{14}C]-stearoyl-CoA and 1-[^{14}C]-palmitoyl-CoA were obtained from NEN Chemicals Ltd. (location, pls) Stearoyl-CoA and palmitoyl-CoA were obtained from Sigma Chemicals, London. Thus urea adduct of methyl sterculate was donated by A.R. Johnson, C.S.I.R.O. Division of Food Preservation, Ryde, N.S.W., Australia. Free sterculic acid which was released from the

[1] Cyclopropenoid fatty acids are named according to the number of carbon atoms in the chain; thus sterculic acid is a C_{18} cyclopropenoid fatty acid.

TABLE I

The Effect of Added Sterculic Acid on the Δ9 Desaturation of Palmitic Acid, Stearic Acid, Palmitoyl-CoA and Stearoyl-CoA in Presence and Absence of the Cofactors for Acyl-CoA Formation[a]

Additions	Percentage desaturation of substrate (% inhibition in brackets)			
	Palmitic acid	Palmitoyl-CoA	Stearic acid	Stearoyl-CoA
NADH, NADPH ATP, CoA, $MgCl_2$	10.4	7.9	9.6	10.0
Sterculic acid (24 μM)	(28.0)	(30.0)	(38.5)	(19.0)
NADH, NADPH	3.1	10.5	1.0	11.3
Sterculic acid (24 μM)		(0)		(0)

[a]All incubations were carried out for 15 min at 30 C in a total volume of 1.25 ml phosphate buffer pH 7.4 containing 0.5 mg microsomal protein from rats fed a high carbohydrate diet. All cofactor concentrations are given in the Materials and Methods.

adduct by the method of James et al. (5) was determined directly from its weight. Ring labeled sterculic acid was prepared by the method of Van Tilborg (7). The free acid was esterified with diazomethane and stored in benzene at -25 C under nitrogen. The radiochemical purity of the methyl sterculate was checked by thin layer chromatography (TLC) on Silica Gel G in the solvent system, 10% diethyl ether in petroleum ether. 1-[^{14}C]-Linoleoyl-CoA was synthesized from the free acid via the N-hydroxysuccinimide ester (8) by the method of Al-arif and Blecher (9). The radiochemical purity was determined, after hydrolysis and methylation, by TLC on Silica Gel G in the solvent system, 50% diethyl ether in petroleum ether. Its chemical purity, which was determined from its absorption at 232 and 260 nm, was in good agreement with the reported values of other coenzyme A esters (10). 5-(2-pentyl-1-cyclopropneyl)- and 5-(2-undecyl-1-cyclopropenyl)-pentanoic acids were synthesized from the corresponding methyl alk-6-ynoates according to the method of Sgoutas and Williams (11,12). The structure of the methylcyclopropenoid esters was confirmed by infrared, proton magnetic resonance, and carbon magnetic resonance spectroscopy and the purity of the methyl esters checked by TLC and by gas liquid chromatography of the silver nitrate-methanol adducts (23).

Animals

Rats were obtained from the Colworth House stocks and, except where stated, were fed a balanced laboratory diet. In certain experiments, it was advantageous to raise the level of the Δ9 desaturase activity by feeding the rats a high carbohydrate diet (13).

Subcellular fractionation and protein determinations were carried out as described by Jeffcoat et al. (13).

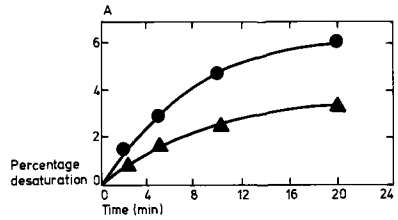

FIG. 1. The effect of sterculoyl-CoA on the time course of the Δ9 desaturase. Incubations contained 86 nmoles stearoyl-CoA, ATP, NADH, NADPH, $MgCl_2$ and CoA (see Materials and Methods) and 2 mg microsomal protein in the absence (●) and presence (▲) of 24 nmoles of sterculate.

Assays for Δ6 and Δ9 Desaturase

Desaturations using free fatty acids as substrates were, unless otherwise stated, carried out at 30 C using mitochondrial supernatants in the presence of 86 nmole fatty acid, 0.16 μmole coenzyme A, 17.5 μmole ATP, 1.1 μmole NADH, 0.48 μmole NADPH, 4 μmole magnesium chloride, and 250 μmole potassium phosphate pH 7.4 in total volume of 1.0 ml. The amount of protein varied from 1.0-5.0 mg and the incubation times are given in the text. Incubations using the coenzyme A esters of the fatty acids were carried out in a similar way with NADH and NADPH as the only added cofactors. Determinations of the fatty acid conversions catalyzed by Δ6 and Δ9 desaturases were carried out as previously described (13).

Gel Electrophoresis

The reversibility of binding of the acyl-CoA to microsomal protein was determined by gel electrophoresis in the presence of sodium dodecylsulphate according to the method of Weber and Osborn (14). The protein bands

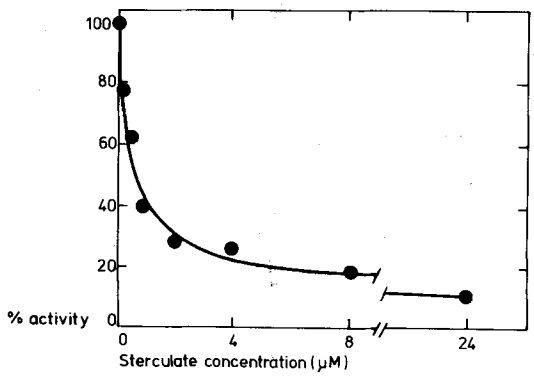

FIG. 2. The effect of sterculoyl-CoA concentration on the activity of the induced Δ^9 desaturase. Incubations contained 0.4 mg microsomal protein, 86 nmoles stearoyl-CoA, cofactors as for Figure 1 and were carried out at 30 C for 15 min with a 6 min pre-incubation in the absence of microsomal protein. 100% activity represented 26.3% desaturation/0.4 g protein/15 min.

TABLE II

The Effect of Added Sterculic Acid[a]

Addition	Percentage desaturation of	
	Linoleic acid	Linoleoyl-CoA
NADH, NADPH ATP, CoA, $MgCl_2$	11.3	9.9
Sterculic acid (24 μM)	(0)	(0)
NADH, NADPH	0	12.0
Sterculic acid (24 μM)	-	(0)

[a]For conditions see Table I on the desaturation of linoleic acid and linoleoyl-CoA.

[b]Magnitude of inhibition is shown in parentheses.

were stained with Coomassie Brilliant Blue R (Sigma Chemical Company, London) and the location of the radioactive material determined by a modification of the method of Tishler and Epstein (15). Approximately 1.5 mm gel sections were digested at 50 C for 45-60 min in a sealed scintillation vial with 0.5 ml 30% hydrogen peroxide and 1 drop of 18 M ammonia. After the gel was cooled, 0.3 ml 4N HCl was added to the digested gel which was then counted in 15 ml 2% (w/v) butyl-PBD in 2:1 (v/v) toluene-Triton X-100.

RESULTS AND DISCUSSION

In Table I we show the effect of 24 μM sterculic acid on the Δ^9 desaturation of palmitic acid, stearic acid, palmitoyl-CoA, and stearoyl-CoA when incubated in the presence and absence of the cofactors required for thiolester formation. The desaturation of palmitic acid and of palmitoyl-CoA is inhibited by about 30% when conditions would allow formation of palmitoyl-CoA and sterculoyl-CoA. The time course of the inhibition is shown in Figure 1. When the conditions prevent formation of the acyl-CoA, then desaturation of palmitic acid is low as expected and the desaturation of palmitoyl-CoA is unaffected. The same is true for stearic acid and stearoyl-CoA. Clearly no inhibition occurs when sterculoyol-CoA cannot be formed and hence it is a safe assumption that sterculoyl-CoA is the inhibitor and not sterculic acid. This is in agreement with earlier indications by other groups (5,16-18).

The inhibitory effect of a range of sterculic acid concentrations is shown in Figure 2. Maximal inhibition is shown at a concentration of about 5 μM at a stearoyl-CoA concentration of 69 μM. This represents an inhibitor-substrate concentration of 1:14. In our experience, it requires much higher ratios of inhibitor to substrate (of the order of 50-100) when other fatty acids are tested as inhibitors (e.g., see Table III). Here inhibition appears to be due to a nonspecific fatty acid effect. In any case, the theory of a detergent effect to account for the marked inhibition of the Δ^9 desaturase cannot explain the specific effect of sterculoyl CoA shown above when sterculic acid is ineffective. Although it could be argued that the lower concentrations of sterculoyl-CoA required to inhibit the stearoyl-CoA desaturase reflect a specificity of this inhibitor, it might also be argued that it reflects the greater denaturating effect of fatty acids coenzyme esters compared with the free fatty acids. This is not likely to be the explanation in this case since Jeffcoat et al. (24) have shown that concentrations of fatty acyl-CoA as high as 100 μM do not inhibit stearoyl-CoA desaturase.

The crude microsomes used to test the inhibition of the Δ^9 desaturase were also used to test the inhibition of the Δ^6 desaturase. In Table II we demonstrate the presence of a Δ^6 desaturase in the preparation by showing conversion of both linoleic acid (provided the cofactors for formation of linoleoyl-CoA are present) and synthetic linoleoyl-CoA.

The results are consistent with the previously reported observations of Brenner (25) that the linoleic acid must first be converted into its coenzyme A ester before it can be desaturated. This implies that the substrate for the Δ^6 desaturase is the coenzyme A or ACP derivative of linoleic acid or some specific linoleic acid containing lipid.

In Table II we demonstrate the effect of added sterculic acid (at the level at which it shows a clear inhibition of the Δ^9 desaturase)

on the desaturation of linoleate and linoleoyl-CoA under similar conditions to those in Table I. The time course of the reaction is shown in Figure 3. No inhibition whatsoever can be found under conditions where both linoleoyl-CoA and sterculoyl-CoA are formed.

In Table III we show the effect of adding the C_{12} cyclopropenoid acid with the cyclopropene ring across carbon atoms 6 and 7, on the conversion of α-linolenic acid (9c, 12c, 15c–18:3) to the C_{18} tetraenoic acid (6c, 9c, 12c, 15c–18:4) at a range of inhibitor-substrate molar ratios. No inhibition could be demonstrated except at such high levels of inhibitor to substrate that one would expect a nonspecific fatty acid effect. In Table III we demonstrate that the same is true for the C_{18} cyclopropenoid fatty acid.

Clearly none of the cyclopropenoid fatty acids are capable of exerting any specific inhibition of the Δ^6 desaturase.

The possible effect of the C_{18} 6,7 cyclopropenoid fatty acid on other desaturation enzymes is shown in Table IV and again it can be seen that effects are observable only at very high inhibitor-substrate ratios unlike the effect of sterculic acid on the Δ^9 desaturase. Of the limited number of cyclopropenoid fatty acids studied to date, only a few are capable of inhibiting a desaturase enzyme and then only the Δ^9 desaturases.

Fogerty et al. (19) and earlier work of the Johnson group (17) have shown that the only effective inhibitors are the following: the C_{17} 8,9-, C_{18} 9,10- and C_{19} 10,11-cyclopropenoid fatty acids. Assuming that the active center of the Δ^9 desaturase falls against the 9,10 methylene groups of the true substrate, then it would follow that a cyclopropenoid fatty acid having the ring involving either or both the C-9 and C-10 atoms could also interact.

It has been demonstrated by Kircher (20) and others (21,22) that cyclopropenoid fatty

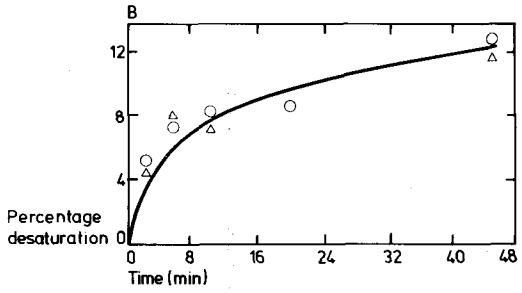

FIG. 3. The effect of sterculoyl-CoA on the time course of the Δ^6 desaturase. Incubations contained 86 nmoles linoleoyl-CoA, the cofactors as in Figure 1 and 10 mg microsomal protein in the absence (○) and presence (△) of 24 nmoles sterculate. All incubations were carried out at 30 C with a pre-incubation time of 6 min in the absence of microsomal protein.

acids, exemplified by sterculic acid, react with thiol groups to form thio-ethers. It has, therefore, been assumed that since the position of the cyclopropene ring in the fatty acid chain is important (19) the inhibition by sterculate is by an interaction with an essential sulphydryl group.

We therefore utilized ^{3}H-sterculic acid (synthesized by Dr. Koch of our Vlaardingen laboratories) as an inhibitor in order to investigate the nature of the enzyme-inhibitor binding. We demonstrate first (Table V) that the [^{3}H]–sterculic acid is as effective an inhibitor as sterculic acid itself. Under conditions which would give ca. 70% inhibition of the stearoyl-CoA desaturase, 750 nmoles [^{3}H]-sterculic acid (7.2 nCi/nmole) were incubated with 100 mg of rat liver microsomal protein obtained from rats fed the high carbohydrate diet. The specific activity of the stearoyl-CoA desaturase was 3.35 nmoles oleic acid produced per min/mg microsomal protein. After a 15 min incubation at 30 C, the activity of the enzyme had been reduced to a specific activity of 1.32. The 25-45% $(NH_4)_2$ SO_4 fraction was prepared as

TABLE III

The Effect of 5-(2-Pentyl-1-cyclopropenyl)- and 5-(2-Undecyl-1-cyclopropenyl)-Pentanoic Acid on the Δ^6 Desaturation of α-Linolenic Acid[a]

Concentration ratio of cyclopropenoic acid to substrate (mole/mole)	5-(2-Pentyl-1-cyclopropenyl)-pentanoic acid		5-(2-Undecyl-1-cyclopropenyl)-pentanoic acid	
	% Conversion	% Inhibition	% Conversion	% Inhibition
0	35	0	35	0
0.01	38.5	0	37	0
0.10	40.0	0	42.5	0
1.0	33.5	4	32.5	7.5
10.0	34.0	2	19.0	41.5
100.0	16.5	53	4.5	87

[a]For conditions see experimental section.

TABLE IV

The Effect of 5-(2-Undecyl-1-cyclopropenyl)-Pentanoic Acid on a Range of Desaturase Enzymes[a]

Substrate	Product	Enzyme involved	Ratio of C_{18} cyclopropenoid fatty acid to substrate	% Conversion	% Inhibition
18:2 (9c 12c)	18:3 (6c 9c 12c)	Δ^6 desaturase	0	14.5	0
			70	1.5	85
20:3 (8c 11c 14c)	20:4 (5c 8c 11c 14c)	Δ^5 desaturase	0	79.5	0
			70	37.2	53
17:0	17:1 (9c)	Δ^9 desaturase	0	74.0	0
			70	53.0	33

[a]Sterculic acid under similar conditions gives an inhibition of 82% of stearic acid desaturation at an inhibitor-substrate ratio of 1.0.

described by Shimakata et al. (26) and the dialyzed material subjected to electrophoresis as described by Weber and Osborn (14) or extracted with chloroform-methanol (27).

After three extractions with the organic solvent, the pellet contained 62 mg protein and 6 nCi [^{3}H]-sterculate. If it is assumed that 0.8% of the protein is desaturase enzyme of which 60% is inhibited by the covalent binding of 1 mole of sterculic acid per mole of enzyme of mol wt 53,000 (28), then the pellet should have contained at least 40 nCi. From similar calculations based on the specific activity of the sterculic acid and the knowledge that 400 μg of protein were applied to the SDS gels, it can be calculated that the desaturase protein band should contain 0.45 nCi or 1000 dpm which in our system is approximately five times background. This apparent lack of label associated with detergent or chloroform-methanol treated protein cannot be reconciled with a covalent binding of inhibitor to substrate. The only tenable conclusion is that the noncovalent binding energy of sterculoyl-CoA to the enzyme is much greater than that of stearoyl-CoA and the clue to the effectiveness must be either in better fit or in some polarization interaction dependent on the cyclopropenoid ring in the vicinity of the C-9 and C-10 atoms of the chain. That the binding is very strong is indicated by the failure of our own group and the Johnson group to show any reversibility of the inhibition by addition of stearic acid (19).

The results all point toward a highly specific and very strong noncovalent attachment of either 8,9-, 9,10-, or 10,11-cyclopropenoid fatty acids that is qualitatively and quantitatively different from the nonspecific fatty acid inhibition postulated by Pande and Meade (6).

In conclusion, therefore, we have been able to establish that whereas sterculic acid is not the inhibitor of stearoyl-CoA desaturase, another derivative, probably its coenzyme A ester, is. Furthermore, in the presence of cofactors required to synthesize the coenzyme A ester, inhibition of Δ^9 fatty acyl-CoA desaturase by this C_{18} cyclopropenoid fatty acid is effected at much lower concentrations than for other fatty acids (see Fig. 2 and Table III). Further, specificity for the inhibition has also been demonstrated by the fact that the "activated" form of sterculic acid is not an inhibitor of the Δ^6 fatty acyl-CoA desaturase. Finally, although it has been suggested that the inhibition by cyclopropenoid fatty acids may occur by covalent interaction with sulphydryl groups on the enzyme, our data provides good evidence that this is not the mode of inhibition of stearoyl-CoA desaturase by the C_{18}

TABLE V

Effect of [^{3}H]-Sterculate on Δ^9 Desaturase Activity[a]

mg Microsomal protein	% Desaturation		% Inhibition
	Without sterculate	With sterculate	
0.18	11.9	3.1	74
0.36	23.1	7.6	67
0.54	32.6	9.6	71

[a]Assays were carried out under the standard conditions described in Materials and Methods using stearoyl-CoA as substrate.

9,10-cyclopropenoid fatty acid. The one remaining question still to be answered is one concerning the identity of the target protein. Is this the cyanide sensitive desaturase protein or some other transferase involved in transporting exogenously supplied stearoyl-CoA to the site of desaturation? The answer to this question can only come from binding studies using purified desaturase and sterculoyl-CoA.

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Influence of Elaidate and Erucate on Heart Mitochondria[1]

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ABSTRACT

Male, weanling rats were fed, for up to six weeks, corn oil (CO), rapeseed oil (RSO), partially hydrogenated fat (HF), or a mixture of partially hydrogenated fat and corn oil (HF + CO). The respiratory activity of their isolated heart mitochondria, their hormone-sensitive lipase activity, and the fatty acid compositions of the phospholipids of the mitochondria were determined. The results indicated that heart mitochondria isolated from rats which had been fed corn oil (CO) had a higher rate of oxygen uptake, showed higher respiratory control ratios, higher ADP/0 ratios and a higher rate of ATP synthesis than the heart mitochondria isolated from those fed rapeseed oil or hydrogenated fats. The oxygen uptake rates of the rat heart mitochondria isolated from each dietary group of rats was in order: oleyl carnitine >> erucyl carnitine > elaidyl carnitine. The decreased capacity to oxidize substrate by heart mitochondria which had been isolated from the hearts of rats fed rapeseed or hydrogenated soybean oil as compared with those fed corn oil as a sole source of dietary fat seemed related to the mitochondria lipid composition. The type of dietary fat fed had a pronounced influence on the mitochondrial fatty acid compositions of phosphatidylcholine, phosphatidylethanolamine, and cardiolipin. The lipase activity of the RSO-fed group did not show any increment with either epinephrine or supplemental ATP treatment. The substrate preference for lipase activity in myocardium was corn oil-triglycerides > trierucin > trielaidin > tripalmitin. However, cardiac lipid accumulation did not seem related to lipase activity in the myocardium.

INTRODUCTION

Rapeseed oil (RSO) as well as partially hydrogenated soybean oil (HF) have caused lipids to accumulate in the myocardium and changed the fatty acid composition of heart mitochondria when fed to weanling rats as a sole source of dietary fat (1-16). The rate of oxygen consumption of heart mitochondria from rats fed rapeseed oil has been reported to be less than the rate of oxygen consumption of heart mitochondria from rats fed sunflowerseed oil (5). Heart mitochondria converted erucic acid to CO_2 more slowly than they did palmitic acid (6), and liver mitochondria converted elaidic acid to CO_2 more slowly than they did oleic acid. However, studies on the oxygen consumption of mitochondria isolated from the hearts of rats fed hydrogenated fat have not been reported to date. Furthermore, whether the cardiac lipid accumulation in rats fed either RSO or HF is related to an increase in triglyceride lipase activity in the heart has not been determined to date. In the present study, the fatty acid composition of heart mitochondrial phospholipid of rats fed either corn oil, rapeseed oil, or hydrogenated fat were determined and the respiratory activity of their heart mitochondria as well as their hormone-sensitive lipase activity was compared.

MATERIALS AND METHODS

(−) Oleyl, erucyl, and elaidylcarnitines were synthesized according to the method of Christophersen and Bremer (17). The purity and identity of the unsaturated acylcarnitines were checked by thin layer chromatography (TLC), gas liquid chromatography (GLC), and mass spectrometry. Oleyl, erucyl, and elaidylchloride were obtained from Sigma Chemical Co., St. Louis, MO). (−) Carnitine chloride was obtained from Koch-Light Ltd., Coln Brook, Bucks, England. Nagarse was obtained from Nagase and Company, Osaka, Japan. ADP, ATP, cytochrome c, pyruvate, L-malate, glutamate, epinephrine, theophylline, trierucin, tripalmitin, bovine serum albumin (fraction V, free of fatty acid), triethanolamine, and gum arabic were purchased from Sigma Chemical Co.

Four groups of 20 male, weanling Holtzman strain rats were fed up to 6 wk a diet[2] con-

[1]Taken from a thesis submitted by Chi Ming Lee Hsu in partial fulfillment of the Ph.D. degree in Food Science, University of Illinois at Urbana-Champaign.

[2]Special fat-free diet was purchased from Nutritional Biochemicals Co., Cleveland, OH; contained in percentage Casein, 29.5; Alphacel Cellulose, 2.0; Sucrose, 64.5; Salt mixture USP XIV, 4.0, plus all vitamin supplements and 0.80 mg vitamin B_{12}.

taining 22% of corn oil[3] (CO), rapeseed oil[4] (RSO), or partially hydrogenated fat[5] (HF). A HF diet at 20% was also supplemented with 2% corn oil (HF + CO) as a source of essential fatty acids. The CO fed group of rats served as controls. Food and water were provided ad libitum. The animals were sacrificed by decapitation at the end of the feeding period. The hearts and livers of the animals within each dietary group were pooled and washed and the mitochondria isolated as described by Pande and Blanchaer (18).

The oxidation rates of acylcarnitines by the isolated mitochondria were determined by measuring the rate of O_2 consumption in an oxygraph with a Clark electrode (Cole-Parmer Co., Chicago, IL). The mitochondria were incubated at 37 C in a medium containing 11 mM K_3PO_4 (pH 7.4); 0.044 mM cytochrome c; 0.22 mM L-malate; 45 mM KCl; 62.5 mM Tris-HCl (pH 7.4); 3.3 mM $MgCl_2$; 7.6 mM sucrose; 5 mg bovine serum albumin; and 0.039 mM acylcarnitine, 0.01 M pyruvate, or 0.01 M glutamate: final volume 3.0 ml. The reaction was started by the addition of 750 nmoles ADP. The rates of oxygen consumption in the presence or absence of ADP were measured and on the basis of these values the rate of O_2 uptake, respiratory control index, and ADP/0 ratio were calculated (5).

Lipolytic enzyme extracts of heart tissue were prepared according to the method of Yammamoto and Drummond (19). Fat emulsions of corn oil[6], tripalmitin, trierucin, and trielaidin were prepared according to published procedures (19). The standard incubation mixtures contained 60 μmoles potassium phosphate buffer, pH 6.8, 5-20 mg bovine serum albumin, the appropriate triglyceride emulsion, 400 μg/ml protamine sulfate, and enzyme extract in a final volume of 1.0 ml. After 3 hr of incubation, the reaction was terminated by adding 10 ml of the Dole extraction mixture. The free fatty acid liberated by the action of the enzyme was assayed by the method of Ho et al. (20).

Lipids were extracted from the mitochondria according to the method of Awasthi et al. (21). The phospholipids were separated by TLC using the two-step system of Skipske and Barclay (22) on 1.0 mm Silica Gel H plates. The first solvent [petroleum ether (bp 30-60 C)-acetone (3:1, v/v)] moved all neutral lipids to the solvent front. The second solvent [chloroform-methanol-acetic acid-water (25:15:4:2, v/v)] separated the phospholipids.

The lipid bands were removed and eluted by a modification of the procedure of Bligh and Dyer (23-25). The samples were esterified with 12% BF-methanol reagent according to the technique of Morrison and Smith (26). Methyl esters were extracted into hexane, dried with anhydrous sodium sulfate, and used directly for GLC analysis.

GLC of the methyl esters was performed with a Varian instrument, model 1400 equipped with a 6 ft glass column, ID 1/8 in., of EGSS-X 10% on Gas Chrom P, 100/120 mesh (Applied Science Lab., Inc., State College, PA), and a flame ionization detector. Helium (carrier gas) flow rate was 40 ml/min at the outlet. The injection port, column and detector temperatures were 270, 180, and 300 C, respectively. Identification of peaks was made by comparison of retention times with those of standard esters run under the same conditions and the percentage of each fatty acid determined by means of triangulation. *trans* Fatty acids were measured quantitatively by GLC with a 12 ft glass column, ID 1/8 in., of Silar 10C on Gas Chrom Q, 100/120 mesh (Applied Science Lab) at 160 C. The free cholesterol level was determined according to the procedure of Sobel and Fernandez (27) and the protein content was determined according to Lowry et al. (28) using serum bovine albumin as the standard.

RESULTS

Heart mitochondria isolated from rats which had been fed corn oil (CO) had a higher rate of oxygen uptake, showed higher respiratory control ratios, higher ADP/0 ratios and a higher rate of ATP synthesis than the heart mitochondria isolated from those fed rapeseed oil or hydrogenated fats (Table I). The oyxgen uptake rates of the rat heart mitochondria isolated from each dietary group of rats were in the order: oleyl carnitine >> erucyl carnitine > elaidyl carnitine. The trend was CO > HF > HF + CO > RSO ($p<0.005$). The rate of ATP synthesis, being the product of twice the rate of O_2-uptake and ADP/0 ratio, showed the same

[3]A gift from the Corn Products Refining Company, Argo, IL. Contained in percentage 15.7, 16:0; 2.3, 18:0; 26.5, 18:1; 55.5, 18:2.

[4]A gift from Agricultural Research Service, Northern Regional Research Lab., Peoria, IL. Contained 5.7, 16:0; 1.4, 18:0; 12.6, 18:1; 14.3, 18:2; 17.0, 18:3; 49.1, 22:1.

[5]A gift from Swift & Company, Oak Brook, IL. Contained in percentage 16.0, 16:0; 10.9, 18:0; 21.4, 18:1 *cis*; 48.6, 18:1 *trans*; 3.1, 18:2.

[6]Corn oil was washed with an alkaline solution, then purified by silicic acid chromatography. The triglycerides fraction was separated from di- and monoglycerides by eluting with 3 to 5% ether in petroleum ether.

TABLE I

The Effect of Dietary Fat on Substrate Oxidates in Isolated Rat Heart Mitochondria

Time in weeks	Substrate	Heart mitochondria isolated from rats fed: Corn oil (CO)	Rapeseed oil (RSO)	Hydrogenated fat (HF)	Hydrogenated fat & corn oil (HF + CO)
One	Oleylcarnitine				
	O_2 uptake rate	231.0 ± 17.0	97.0 ± 1.2[Ca]	145.3 ± 5.8[C]	116.6 ± 1.5[C]
	RCR[b]	7.1	4.1	5.6	6.7
	ADP/0	3.2	2.9	2.9	2.9
	ATP syn. rate[c]	1478.4	562.6	842.7	676.3
Two	O_2 uptake rate	313.6 ± 16.7	216.8 ± 31.9[C]	255.7 ± 16.2[C]	221.3 ± 16.1[C]
	RCR	8.7	4.7	5.9	6.0
	ADP/0	3.1	2.4	2.9	2.7
	ATP syn. rate	1944.3	1040.6	1483.1	1195.0
Four	O_2 uptake rate	376.2 ± 18.8	284.3 ± 2.9[C]	342.7 ± 21.3[C]	264.0 ± 39.4[C]
	RCR	8.9	4.2	6.1	4.4
	ADP/0	3.2	3.2	3.2	2.8
	ATP syn. rate	2407.7	1819.5	2193.3	1478.4
Six	O_2 uptake rate	257.6 ± 11.3	258.5 ± 23.8[D]	230.8 ± 21.6[C]	234.4 ± 15.3[C]
	RCR	8.6	4.4	5.9	6.9
	ADP/0	3.4	2.8	3.4	3.1
	ATP syn. rate	1751.7	1447.6	1569.4	1453.3
	Erucylcarnitine				
One	O_2 uptake rate	110.7 ± 4.5	62.7 ± 3.5[C]	73.3 ± 12.9[C]	66.6 ± 14.2[C]
	RCR	5.6	2.8	3.7	3.4
	ADP/0	3.5	3.0	3.4	3.2
	ATP syn. rate	774.9	376.2	498.4	426.2
Two	O_2 uptake rate	173.2 ± 11.3	119.0 ± 11.0[C]	143.8 ± 27.6[C]	122.8 ± 7.8[C]
	RCR	5.3	3.93	4.6	3.8
	ADP/0	3.5	2.7	3.3	2.9
	ATP syn. rate	1212.4	642.6	949.1	712.2
Four	O_2 uptake rate	252.3 ± 36.3	185.7 ± 6.8[C]	205.9 ± 12.2[C]	208.3 ± 13.4[C]
	RCR	5.82	3.02	4.63	4.0
	ADP/0	2.5	2.3	2.2	2.2
	ATP syn. rate	1261.5	854.2	906.0	916.5
Six	O_2 uptake rate	179.5 ± 14.3	163.1 ± 9.5[C]	157.6 ± 7.4[C]	156.7 ± 3.9[C]
	RCR	5.4	4.5	4.8	5.0
	ADP/0	3.5	2.9	3.6	3.8
	ATP syn. rate	1256.5	946.0	1134.7	1190.9
	Elaidylcarnitine				
One	O_2 uptake rate	91.0 ± 4.0	44.7 ± 7.7[C]	89.6 ± 2.3[A]	57.3 ± 5.1[C]
	RCR	6.2	3.6	3.2	4.2
	ADP/0	3.0	2.5	2.6	2.7
	ATP syn. rate	546.0	223.5	465.9	309.4
Two	O_2 uptake rate	137.4 ± 7.3	98.2 ± 9.9[C]	125.4 ± 12.2[C]	115.4 ± 17.5[C]
	RCR	3.4	3.1	3.5	4.2
	ADP/0	3.1	2.5	2.9	2.5
	ATP syn. rate	851.9	491.0	727.3	577.0
Four	O_2 uptake rate	227.20 ± 7.5	160.6 ± 8.8[C]	192.4 ± 0[C]	184.9 ± 10.7[C]
	RCR	4.4	2.9	3.2	3.5
	ADP/0	2.3	1.6	1.4	2.1
	ATP syn. rate	1045.1	513.9	538.7	776.6
Six	O_2 uptake rate	171.6 ± 42.8	125.5 ± 12.3[C]	154.0 ± 4.0[A]	144.0 ± 3.2[A]
	RCR	4.5	3.6	4.4	4.9
	ADP/0	3.2	3.2	3.0	3.2
	ATP syn. rate	1098.2	803.2	924.0	921.6
	Pyruvate				
One	O_2 uptake rate	290.7 ± 14.2	134.2 ± 19.3[C]	263.7 ± 2.1[C]	270.7 ± 15.4[C]
	RCR	7.8	5.0	7.9	6.6
	ADP/0	2.9	2.6	2.5	2.2
	ATP syn. rate	1686.1	697.8	1318.5	1191.1

TABLE I (Cont.)

The Effect of Dietary Fat on Substrate Oxidates in Isolated Rat Heart Mitochondria

Time in weeks	Substrate	Heart mitochondria isolated from rats fed: Corn oil (CO)	Rapeseed oil (RSO)	Hydrogenated fat (HF)	Hydrogenated fat & corn oil (HF + CO)
Two	O_2 uptake rate	276.4 ± 14.2	199.2 ± 7.5[C]	214.3 ± 10.3[C]	195.6 ± 3.3[C]
	RCR	8.3	5.3	5.8	6.8
	ADP/0	2.3	2.2	2.1	2.1
	ATP syn. rate	1271.4	876.5	900.0	821.5
Four	O_2 uptake rate	387.3 ± 15.6	240.9 ± 6.8[A]	352.4 ± 11.0[C]	192.2 ± 17.5[C]
	RCR	8.1	3.8	5.5	3.4
	ADP/0	3.1	2.8	3.5	3.0
	ATP syn. rate	2401.3	1349.0	2466.8	1153.2
Six	O_2 uptake rate	292.1 ± 9.5	268.5 ± 0.0[C]	253.8 ± 2.0[C]	254.8 ± 15.4[C]
	RCR	8.2	6.0	6.2	6.5
	ADP/0	3.4	3.0	3.2	3.2
	ATP syn. rate	1986.3	1611.0	1624.3	1630.7
	Glutamate				
One	O_2 uptake rate	164.4 ± 3.4	25.7 ± 2.0[C]	136.5 ± 13.4[C]	139.8 ± 2.1[C]
	RCR	6.3	3.7	5.5	6.6
	ADP/0	5.3	13.5	4.9	4.5
	ATP syn. rate	1742.6	693.9	1337.7	1258.2
Two	O_2 uptake rate	182.3 ± 9.3	38.0 ± 1.8[C]	174.8 ± 12.1[A]	156.4 ± 7.1[C]
	RCR	8.3	3.0	5.6	6.1
	ADP/0	4.6	10.7	4.8	4.3
	ATP syn. rate	1677.2	813.2	1678.1	1345.0
Four	O_2 uptake rate	389.5 ± 18.8	173.5 ± 0.0[C]	244.5 ± 11.0[C]	295.4 ± 2.1[C]
	RCR	7.0	3.5	5.9	5.8
	ADP/0	4.0	7.6	3.8	3.7
	ATP syn. rate	3116.0	2637.2	1858.2	2186.0
Six	O_2 uptake rate	247.9 ± 7.5	183.0 ± 19.4[C]	211.6 ± 10.2[C]	193.2 ± 1.9[C]
	RCR	7.3	3.6	7.2	7.4
	ADP/0	3.4	3.9	3.4	3.4
	ATP syn. rate	1685.7	1424.7	1438.9	1313.8

[a]Results are means ± SD of three determinations (expressed as nmoles/min/mg protein). A, B, C are significantly different from the CO values: $P<0.05$, $P<0.01$ and $P<0.005$, respectively. D: not significantly different from the CO values.

[b]Respiratory control ratio.

[c]Rate of ATP synthesized = ADP/0. Rate of O_2 uptake x 2; expressed as nmoles/min/mg protein.

trend. The differences were pronounced at the first week, but became much less so at the end of 6 wks.

The oxygen uptake rates of rat liver mitochondria isolated from the rats fed rapeseed oil or hydrogenated fat did not differ significantly from those isolated from rats fed corn oil (Table II). However, differences in the oxygen uptake rate of the isolated mitochondria in the presence of oleyl carnitine, erucyl carnitine, or elaidyl carnitine as substrate showed the same trend as heart mitochondria, i.e., oleyl carnitine > erucyl carnitine > elaidyl carnitine. The ratio of oxidation rates by liver mitochondria of erucyl carnitine as compared to elaidyl carnitine was nearly 2 to 1 whereas the ratio of oxidation rates by heart mitochondria was 1.2 to 1.

The mitochondria isolated from phospholipids of rats fed either rapeseed oil (RSO), hydrogenated fat (HF) or hydrogenated fat plus corn oil (HF + CO) had altered fatty acid compositions compared to those fed corn oil (CO) (Table III). At one week, there was a relatively greater amount of palmitoleic (16:1) acid in the phosphatidylethanolamine (PE) from animals fed RSO. Compared to those fed CO, there were consistently greater amounts of oleic acid (18:1) and lesser amounts of linoleic acid (18:2) and arachidonic acid (20:4) in the phospholipid fractions from animals fed RSO. Eicosenoic acid (20:1) was found to some extent in all phospholipid fractions. Larger amounts of 20:1 were found in the phosphatidylcholine (PC) and cardiolipin (CL) fractions of animals fed RSO than in animals fed CO.

TABLE II

The Effect of Dietary Oils on Substrates Oxidation in Isolated Rat Liver Mitochondria[a]

	CO	RSO	HF	HF + CO
1 wk				
Oleylcarnitine	42.5	52.9	53.1	54.6
Erucylcarnitine	26.8	33.5	29.4	31.1
Elaidylcarnintine	15.2	18.0	14.7	15.9
Pyruvate	38.4	43.5	36.8	41.1
Glutamate	20.9	22.3	23.9	20.5
2 wk				
Oleylcarnintine	43.2	39.7	44.0	43.6
Erucylcarnintine	33.5	19.8	33.8	25.8
Elaidylcarnitine	10.7	16.3	10.4	12.8
Pyruvate	49.2	47.8	53.0	43.7
Glutamate	29.3	26.2	32.2	23.9
4 wk				
Oleylcarnitine	74.3	78.5	86.0	87.6
Erucylcarnitine	46.9	48.0	62.6	54.4
Elaidylcarnitine	16.3	19.5	27.5	19.8
Pyruvate	41.8	49.2	64.9	51.9
Glutamate	41.8	42.1	59.2	48.5
6 wk				
Oleylcarnitine	69.6	71.7	58.9	85.1
Erucylcarnitine	38.5	43.6	33.6	59.5
Elaidylcarnitine	27.8	52.8	28.4	43.3
Pyruvate	61.7	63.1	50.8	68.6
Glutamate	52.4	64.2	55.0	62.2

[a]Results are expressed as an average of three determinations (expressed as nmoles/min/mg protein). See Table I for identification of abbreviations.

The influences of the HF and the HF + CO diets on the fatty acid pattern in the CL, PE, and PC from rat heart mitochondria were similar to those for rats fed RSO; marked decreases in 18:2, 20:4, and 22:6 and increases in 16:1, 18:1, and 20:3 fatty acids were noted. The only exception was that 20:4 of PC from the HF + CO group was almost equal to that of the CO group.

There was considerable incorporation of dietary elaidic acid into all three phospholipid fractions. The *trans* isomer represented nearly half of the total octadecenoic acid. The differences between the rats fed HF + CO or CO were not as pronounced as the differences between rats fed HF or CO. However, supplemented corn oil seemed to have little effect on the amount of elaidic acid incorporated into the phospholipids. The *trans* fatty acid content of the CO supplemented HF rats was not significantly different from that of the rats fed only HF. Unlike erucic acid, which seemed incorporated in CL and equally well in PC, elaidic acid incorporated more readily into the CL fraction of the HF and HF + CO fed groups. The differences in the mitochondrial fatty acid compositions between groups were not so pronounced at the sixth week.

The cholesterol levels in heart and liver mitochondria of rats (in μg cholesterol/mg protein) increased during the 6 wk feeding period (Table IV). The cholesterol content of heart mitochondria isolated from the rats which had been fed either RSO or HF + CO was significantly lower than that of the CO group at the first week or 2.5, 2.8 and 3.9 μg, respectively. At the end of 6 wk, both the RSO and the HF + CO fed group contained higher heart mitochondria cholesterol levels than the CO group or, 5.1, 5.5 and 4.9 μg, respectively. The HF fed group, on the other hand, had a heart mitochondria cholesterol level of 3.6 μg at one week and 4.4 μg at 6 wk. The cholesterol levels of liver mitochondria isolated from the rats which had been fed corn oil were significantly higher than those from the other dietary groups after both 1 and 6 wk on the diet.

The myocardial hormone-sensitive lipase (or triglyceride lipase) activity from rats fed CO, RSO, HF or HF + CO for up to 6 wk, with pretreated corn oil[6] as substrate, is given in Table V. The incubation time was set at 3 hr. to increase the amount of free fatty acid released. The basal myocardial lipase activity did not show a consistent increase or decrease with time. Furthermore, the relative activity between groups did not show any general trend. At the first week of feeding, the lipase activity of the CO fed group was highest, whereas that of the HF + CO fed group was lowest among the four dietary groups studied. After 6 wk, lipase activity in the rats fed HF + CO was highest and that of the RSO fed group was lowest; but the lipase activity of the HF fed group was still lower than of the rats fed CO.

The activity of myocardial enzyme was increased 30% by epinephrine treatment. When the epinephrine-treated tissue was supplemented with ATP, the activity of enzyme was increased 55.5% for the CO fed group. Activation by epinephrine, as well as epinephrine with ATP, increased the enzyme activity for the HF fed group by 41% and 78% and that for the HF + CO fed group by 26% and 97%, respectively. After 6 wk, the activation differences between diets became less pronounced, except those fed RSO still showed significantly lower increment of enzyme activity after stimulation with added epinephrine or epinephrine supplemented with ATP. The myocardial lipase activity toward substrates other than corn oil was lower (Table VI). In general, with trierucin as substrate, the enzyme showed a higher hydrolysis rate than with trielaidin, which in turn was a better substrate for the enzyme than the tripalmitin.

TABLE III

Relative Composition[a] of Total Fatty Acids of Separated Phospholipids in Pooled Rat Heart Mitochondria from Rats Fed Corn Oil, Rapeseed Oil or Hydrogenated Fat

	Corn oil			Rapeseed oil			Hydrogenated fat			Hydrogenated fat plus corn oil		
One wk	CL	PE	PC[b]	CL	PE	PC	CL	PE	PC	CL	PE	PC
16:0[c]	1.4	10.6	19.7	16.4	10.4	15.3	12.4	20.1	20.1	9.9	15.0	13.6
16:1ω7	1.2	1.0	2.7	1.5	4.1	1.7	2.9	1.5	3.6	1.1	0.4	1.6
18:0	2.8	22.3	24.5	5.9	19.0	26.2	7.5	23.2	23.2	5.1	22.0	25.5
18:1-*cis*ω9	14.0	19.2	15.5	25.1	29.6	30.1	21.1	18.5	19.2	14.2	15.3	13.2
18:1-*trans*ω9	-	-	-	-	-	-	21.5	18.5	17.0	18.0	12.0	13.3
18:2ω6	68.3	16.2	21.2	38.7	12.3	8.5	29.2	1.7	4.6	44.4	7.1	9.2
18:3ω3	0.3	0.1	0.1	1.3	0.9	0.6	-	-	-	0.1	0.2	-
20:1ω11	2.1	0.5	1.6	4.7	3.6	4.3	0.8	-	-	-	-	-
20:3	1.7	0.8	0.5	0.7	1.8	1.8	2.0	2.3	3.5	2.8	1.7	1.3
20:4ω6	2.6	14.8	11.4	0.9	6.1	4.1	0.1	6.4	5.5	0.3	9.9	15.3
22:1ω9	0.2	0.1	0.1	3.8	1.6	2.9	-	-	-	-	-	-
22:6	5.0	14.3	5.2	1.1	15.4	4.5	2.5	8.9	3.2	4.4	16.3	7.0
Six wk												
16:0	1.2	6.8	10.4	5.1	8.8	7.5	4.6	11.5	19.2	2.8	6.8	9.8
16:1ω7	2.2	0.6	0.7	1.7	1.9	1.8	5.0	1.3	1.6	1.1	0.4	0.5
18:0	4.5	37.3	36.0	5.2	19.7	36.3	6.2	34.4	27.6	4.8	31.8	31.3
18:1-*cis*ω9	10.4	15.5	14.7	24.1	29.4	20.0	22.4	25.3	26.6	16.0	15.8	16.1
18:1-*trans*ω9	-	-	-	-	-	-	17.4	28.8	21.6	17.2	14.4	14.0
18:2ω6	72.3	11.8	14.6	55.9	12.7	11.2	41.2	7.0	10.6	52.1	11.1	11.7
18:3ω3	0.1	0.1	-	0.5	0.1	0.2	-	-	-	0.6	0.4	0.5
20:1ω11	1.2	0.1	0.9	2.1	0.2	1.7	0.8	0.2	0.1	-	-	-
20:3	1.5	0.6	0.9	0.8	0.5	0.9	1.9	0.9	1.5	1.5	0.8	1.0
20:4ω6	2.6	21.6	20.3	2.0	21.1	17.5	0.5	6.0	7.6	1.4	11.9	13.4
22:1ω9	0.4	0.1	0.1	2.2	0.7	0.8	-	-	-	-	-	-
22:6	3.3	5.5	1.4	0.4	4.9	0.6	0.4	3.4	0.6	3.0	6.5	1.7

[a]Values are expressed as area percent of the total peak areas. Results are the average of triplicate gas chromatography analysis.

[b]CL - Cardiolipin, PE - Phosphatidylethanolamine, PC - Phosphatidylcholine.

[c]Carbon atom chain length: number of double bonds.

TABLE IV

Effect of Dietary Fat on Heart and Liver Mitochondrial Cholesterol Content[a]

		CO	RSO	HF	HF + CO
Heart					
	1 wk	3.9 ± 0.3	2.5 ± 0.4[C][b]	3.6 ± 0.1[C]	2.8 ± 0.1[C]
	2 wk	4.0 ± 0.2	3.4 ± 0.3[C]	3.9 ± 0.1[A]	3.1 ± 0.3[C]
	4 wk	4.5 ± 0.6	4.4 ± 0.1[D]	4.2 ± 0.2[A]	4.7 ± 0.5[D]
	6 wk	4.9 ± 0.4	5.1 ± 0.3[A]	4.4 ± 0.1[C]	5.5 ± 0.2[C]
Liver					
	1 wk	5.1 ± 0.2	2.7 ± 0.1[C]	2.0 ± 0.1[C]	3.3 ± 0.3[C]
	2 wk	5.0 ± 0.1	3.0 ± 0.0[C]	2.4 ± 0.0[C]	3.9 ± 0.4[C]
	4 wk	5.3 ± 0.3	3.8 ± 0.1[C]	2.7 ± 0.2[C]	4.1 ± 0.0[C]
	6 wk	5.4 ± 0.5	4.1 ± 0.2[C]	3.5 ± 0.4[C]	4.9 ± 0.2[C]

[a]Results are means ± SD of three determinations; expressed as μg cholesterol/mg protein.

[b]A, B, C significantly different from the CO values: $P<0.05$, $P<0.01$ and $P<0.005$, respectively. D: not significantly different from the CO values.

DISCUSSION

The decreased capacity to oxidize substrate by heart mitochondria which had been isolated from the hearts of rats fed rapeseed or hydrogenated soybean oil as compared with those fed corn oil as a sole source of dietary fat seemed related to their lipid composition. Houstmuller et al. (5) and Christophersen and Bremer (8) have shown that this decreased capacity of

TABLE V

Myocardial Hormone-Sensitive Lipase Activity[a] of Rats on Dietary Lipids for up to 6 Wk[c]

	6 wk			2 wk			4 wk			6 wk		
	None	Epinephrine	ATP + Epinephrine	None	Epinephrine	ATP + Epinephrine	None	Epinephrine	ATP + Epinephrine	None	Epinephrine	ATP + Epinephrine
CO	250.0	325.2 (30)[b]	388.8 (55.5)	167.4	252.6 (50.9)	252.6 (50.9)	176.9	217.6 (23)	249.4 (41)	306.5	404.6 (32)	462.8 (51)
RSO	214.3	205.9 (0)	228.1 (6.67)	215.7	203.4 (0)	228.6 (5.9)	93.8	97.1 (3.6)	105 (12)	122.2	128.8 (5.4)	135.6 (11)
HF	215.7	304.2 (41)	384.0 (78)	126.3	170.8 (35)	275.5 (118)	192.7	229.3 (19)	296.8 (54)	268.5	346.4 (29)	421.5 (57)
HF + CO	192.8	242.9 (26)	379.8 (97)	123.7	174.4 (41)	267.3 (116)	191.7	241.5 (26)	308.6 (61)	358.9	484.6 (35)	495.3 (38)

[a]Activity expressed as n equiv. FFA released/mg protein/3 hr. Substrate used was corn oil which has been pretreated to remove FFA, MG and DG.
[b]Values in parentheses are percentage increased by activation.
[c]Tissues from 14 rats in each group were pooled and homogenized. Values given for each group are the average of three determinations made on one pooled sample.

heart mitochondria to oxidize substrate did not apply to liver mitochondria. This observation was confirmed in the present study. Houstmuller et al. (5) reported that the rate of oxygen uptake and the rate of ATP synthesis in heart mitochondria isolated from the hearts of rats which had been fed sunflowerseed oil was three times higher than in those fed rapeseed oil. Similar results were obtained in the present study in the heart mitochondria isolated from rats fed corn as compared to those fed rapeseed oil or hydrogenated fat. As heart mitochondria contain an even higher proportion of highly unsaturated fatty acids than liver mitochondria, heart mitochondria may be more sensitive to an influx of unnatural unsaturated fatty acids.

Previous studies by Kramer et al. (10), Lands et al. (29), and Egwim and Sgoutas (12) indicated that erucic or elaidic acid or their elongated derivatives were preferentially incorporated at the two position in phosphatidylcholine when each was fed as a main dietary fat source. In the presence of linoleic acid, elaidic acid was preferentially incorporated at the one position in phosphatidylcholine (29). When phospholipids which contain erucic or elaidic acid or their elongated derivatives are incorporated into cell membranes, their permeability (30) or enzyme binding sites may be changed sufficiently to allow triglycerides to accumulate in the heart tissue.

The lipid components of the lipid classes in the lipoproteins isolated from the plasma of rats which had been fed hydrogenated soybean oil contained from 6 to 39% elaidic acid (31), and over 50% of the total 18:1 in their heart tissue was in the *trans* configuration (32). When rapeseed oil or hydrogenated soybean oil served as the only source of dietary fat, the plasma lipoprotein reflected their fatty acid composition. Weanling animals have no choice but to incorporate these fatty acids into their heart mitochondria. The rapidity at which the rat adjusts to the presence of these fatty acids in the plasma lipoproteins indicates that, in the absence of a dietary source of linoleic acid, the linoleic acid from depot fat and other tissue replaces erucic or elaidic acid in the phospholipids of heart mitochondria.

Cholesterol plays an important role in controlling the rigidity of membrane lipids. Previous observation (33-36) strongly suggest that cholesterol can cause a dual effect on phospholipids, depending on the nature of their fatty acid constituents, as the interaction of cholesterol with saturated and unsaturated phospholipids in the membrane system mediates the degree of membrane fluidity (30). The present

TABLE VI

Myocardial Hormone-Sensitive Lipase Activities Toward Tripalmitin, Trierucin, and Trielaidin as Substrates

Diet fat	1 wk			6 wk		
	Tripalmitin	Trierucin	Trielaidin	Tripalmitin	Trierucin	Trielaidin
Corn oil	23.6	83.1	47.6	29.2	101.6	53.4
Rapeseed oil	22.9	79.5	-	10.4	42.9	-
Hyd. fat	20.0	-	35.4	23.2	-	50.9
Hyd. fat + corn oil	15.4	-	33.0	30.2	-	82.9

[a]Activity expressed as n equiv. FFA/mg protein/3 hr. For experimental details: See footnote c to Table V.

results indicated that the mitochondrial cholesterol content of heart tissue from rats fed HF + CO, HF, and RSO was significantly lower as compared to the tissue from rats fed CO. The ratio of cholesterol to phospholipid in mitochondrial membrane of the RSO, HF, and HF + CO groups at the first week of feeding may have been inadequate for proper membrane fluidity.

The present results indicated that erucic acid and elaidic acid seemed to have an affinity for the cardiolipin molecule of the rat heart mitochondria. It is possible that the fatty acid compositional change of membrane cardiolipin may have some effect on the activity of membrane bound enzymes such as cytochrome oxidase. The respiratory control ratio and the ADP/0 ratio can be used as indicators of mitochondrial structural integrity. Since heart mitochondria of the CO fed group showed high respiratory control ratios, these mitochondrial preparations should give a good coupling of oxidation to phosphorylation. The lower respiratory control and ADP/0 ratios of the RSO and HF fed rats might, therefore, reflect the malfunctioning of some enzyme systems. The change was more marked for the NAD-linked dehydrogenase systems for glutamate and malate, and less pronounced for the inner membrane enzyme systems such as respiratory chain enzymes, ATP-synthesizing enzymes, and carnitine fatty acyltransferase. As cytochrome oxidase can be used as a marker for the mitochondrial inner membrane, glutamate and malate dehydrogenase also served as markers for the matrix which is surrounded by the inner membrane. The prominent impairment of respiratory activity in the mitochondria of RSO-fed rats with respect to glutamate and malate suggests that the fatty acid compositional change of the matrix had influenced biological functions. Trump et al. (37) stated that the loss of the ability of mitochondria to synthesize ATP seems to represent "the principal limiting factor in determining the time of cell survival."

It has been noted in previous studies that RSO-fed animals showed enlargement in the thyroid gland and adrenal gland (1), thus indicating a possible hormone imbalance in the animals. Maude et al. (38) recently pointed out that the fat cells from hypothyroid rats do not give a lipolytic response to catecholamines but give a response to the cyclic AMP analog, N^6, $O^{2'}$-dibutyryl-3',5'-cyclic adenylate. Maude et al. considered this analog to act primarily upon protein kinase and bypassed the adenylate cyclase activation step in the sequence of events leading from epinephrine stimulation to an active triglyceride lipase. The addition of epinephrine alone also increased activity which implies that, regardless of the lower level of membrane linoleic acid content of the HF and HF + CO fed rats, the adenylate cyclase still functioned normally. This finding did not agree with Carreau's assumption (39) that a correlation existed between linoleic acid concentration and hormone-sensitive lipase activity. The lower availability of ATP may have resulted in lower 3',5'-cyclic AMP transformed, which in turn affected the lipolytic activity of the myocardial tissue obtained from the rats fed HF or HF and CO.

One would expect that trierucin would be the best substrate for triglyceride lipase because its melting point is below body temperature, whereas tripalmitin (mp α44.7, β56.6, 66.4 C) and trielaidin (mp α16.6, β42.8 C) have melting points above body temperature. Therefore, at 37 C, tripalmitin and trielaidin tend to separate from the emulsion. The melting points of the substrates are in the order: tripalmitin > trielaidin > trierucin; the lipase activity toward the substrates was in a reverse order: corn oil > trierucin > trielaidin > tripalmitin. Our results did not agree with those reported by Kramer et al. (10) who showed a negligible trierucin hydrolysis by rat heart triglyceride lipase.

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Effect of Dietary Lipids on Fatty Acid Composition of Body Lipid in Rainbow Trout (*Salmo gairdneri*)[1]

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ABSTRACT

Three isocaloric diets were prepared. Diet 1 (Control) contained 22% herring oil. In diets 2 and 3, a third and a half of the herring oil was replaced, respectively, by an animal fat (lard) which contained a high percentage of saturated fatty acids. Each diet was fed to duplicate groups of rainbow trout for 14 wk. The results of the feeding trial indicated that the concentration of the saturated fatty acids in trout body lipid did not increase despite the high concentration of these fatty acids in Diets 2 and 3. Fish growth, feed efficiency, mortality and the level of fatty acid deposited in fish body lipid and phospholipids are discussed.

INTRODUCTION

Many commercial fish rations as well as experimental fish diets are generally low in lipid content, rarely exceeding 10-12% on a dry weight basis. Problems of fat stability leading to autoxidation of lipid and attendent vitamin destruction which can occur during storage as well as the difficulty of handling a high fat food are plausible reasons for the low levels. In addition, high fat diets have occasionally been held responsible for various physiological disorders. Recent studies in our laboratory and by others have demonstrated the value of increasing the energy level of the diet by lipid incorporation and particularly the addition of ω3 fatty acids (FAs), both of which produce improved growth and well-being. Vegetable oils, many of which contain a high proportion of ω6 FAs, however, have been shown to retard growth (1,2). Lee and Putnam (3) reported that a part of the dietary protein was spared when the energy density of fish diet was increased by adding fat. Animal fats, derived from mammals, could serve as a good source of energy, but are more saturated than vegetable or fish oils and usually are low in ω6 FA and in the ω3 FAs, the latter

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TABLE I

Percentage Composition of Diets and Growth Response of Fish[a]

Ingredients	Diet no. 1	2	3
Protein[b]	33.33	33.33	33.33
Herring oil	22.00	14.60	11.00
Lard	0	7.40	11.00
Dextrin	26.00	26.00	26.00
Cellulose	11.17	11.17	11.17
Premix[c]	7.50	7.50	7.50
Average initial fish weight (g)	5.40	5.40	5.41
Average final fish weight (g)	27.5	28.1	27.8
Feed conversion efficiency (wt gain/feed consumed)	0.94	0.96	0.90
Fat in feed consumed (g)	248.6	248.2	264.2
Fat deposited in fish (g)	100.7	106.2	105.8
% Lipid in fish at termination	9.45	9.85	9.80
Accumulated mortality (%)	3	6	3

[a]Average values of duplicate groups.

[b]Protein contained 75% casein + 25% gelatin.

[c]Premix (% in diet): mineral mix, 4.00; vitamin mix, 2.00; methionine, 0.20; tryptophane, 0.10; vitamin E concentrate, 0.20 (660 IU/kg); choline chloride, 1.00; total = 7.50%.

Mineral mix: Bernhart-Tomarelli salt mix. Vitamin mix: supplies (mg/kg of diet): thamin, 64; riboflavin, 144; niacinamide, 512; biotin, 1.6; Ca-pantothenate, 288; pyridoxine, 48; folic acid, 19.2; menadione, 16; cobalamine, 0.16; *i*-inositol, 2500; ascorbic acid, 1200; PABA, 400; vitamin D_2, 4000 IU/kg; vitamin A, 25,000 IU/kg.

TABLE II

Percentage Fatty Acid (FA) Composition of Dietary Lipids

FA	Diet no. 1	2	3
14:0	4.7	3.6	3.1
16:0	12.9	17.6	20.0
16:1	7.6	5.7	4.7
16:2[a]	1.2	0.8	0.7
18:0	3.0	7.4	9.6
18:1ω9	25.3	30.5	33.1
18:2ω6	1.4	4.5	6.0
18:3ω3	0.6	0.7	0.8
20:1ω9	9.2	6.5	5.3
18:4ω3	3.0	2.0	1.5
20:4ω6	0.5	0.3	0.3
22:1ω13+ω11	8.8	5.9	4.4
20:4ω3	1.1	0.7	0.6
20:5ω3	8.7	5.8	4.4
22:5ω6	1.0	0.7	0.5
22:5ω3	1.6	1.1	0.8
22:6ω3	9.4	6.2	4.7
Total ω3	24.4	16.5	12.8
Total ω6	2.9	5.5	6.8
Total saturated FAs	20.6	28.6	32.7
Total monoenoic acids	50.9	48.6	47.5

[a]May also contain 17:1.

which are desirable in fish rations.

The metabolism of saturated animal fats by fish has not been thoroughly studied, but it is generally agreed that high levels would probably be undesirable if the end result was a body fat of greater saturation. Such has been the case with swine (4,5), rat (6), and poultry (7). Reiser et al. (8) pointed out that marine and freshwater fish probably do not differ from other classes of animals in the mechanism of deposition and interconversion of dietary FAs. This paper describes the FA composition of the body lipid of three groups of rainbow trout fed various levels of saturated FAs. The control group was fed a semipurified diet containing 22% herring oil. The second and third groups were fed the same diet except that a part of the dietary herring oil was replaced by the more saturated animal fat, lard.

MATERIALS AND METHODS

Three isocaloric diets were prepared by using the same diet ingredients. The percentage concentrations of protein, dextrin, lipid, and other minor nutrients were the same in the three diets. Diet 1 (control) contained 22% herring oil. In diets 2 and 3, however, 33% and 50% of the herring oil was replaced, respectively, by lard. The composition of the three diets is shown in Table I.

Rainbow trout fry from our brood stock were fed for 4 mo with a casein-gelatin-dextrin-

TABLE III

Percentage Fatty Acid (FA) Composition of Trout Whole Body Total Lipid and Phospholipids[a]

FA	Body total lipid Diet no. 1	2	3	Phospholipids Diet no. 1	2	3
14:0	4.4	3.5	3.5	2.1	2.4	2.3
16:0	16.2	16.7	16.8	16.8	18.6	19.7
16:1ω7[b]	9.8	8.4	7.8	3.5	3.6	4.3
16:2[c]	0.8	0.6	0.6	0.4	0.1	0.3
18:0	3.3	3.8	4.4	4.6	5.4	5.8
18:1ω9[b]	31.0	37.3	41.9	17.8	19.7	21.3
18:2ω6	1.4	4.7	6.9	0.5	1.8	3.1
18:3ω3	0.9	0.9	0.7	0.1	0.1	0.3
20:1ω9[b]	6.5	5.5	4.4	2.1	1.5	1.5
18:4ω3	2.3	2.0	1.5	--	--	--
20:4ω6	0.7	0.2	0.4	1.2	1.4	1.2
22:1ω13+ω11	4.0	2.2	2.0	1.4	0.9	0.5
20:4ω3	0.9	0.5	0.4	--	--	--
20:5ω3	5.3	3.8	2.9	9.2	8.3	5.8
22:5ω3	1.5	1.4	0.3	3.7	1.8	2.6
22:6ω3	11.1	8.6	5.6	36.5	34.6	31.4
Total ω3 FAs	22.0	17.2	11.4	49.5	44.8	40.1
Total ω6 FAs	2.1	4.9	7.3	1.7	3.2	4.3
Total saturated FAs	23.9	24.0	24.7	23.5	26.4	27.8
Total monoenoic acids	51.3	53.4	56.1	24.8	25.7	27.6

[a]Average of analysis of duplicate groups.

[b]Other isomers may be present.

[c]May also contain 17:1.

TABLE IV

Quantity of Fatty Acid (FA) from Diets and Amount Deposited in Fish

FA	Diet 1		Diet 2		Diet 3	
	FAs from diet (g)	FAs deposited in fish (g)	FAs from diet (g)	FAs deposited in fish (g)	FAs from diet (g)	FAs deposited in fish (g)
14:0	11.7	4.4	8.9	3.7	8.2	3.7
16:0	32.1	16.3	43.7	17.7	52.8	17.8
16:1	18.9	9.9	14.2	8.9	12.4	8.2
16:2[a]	3.0	0.8	2.0	0.6	1.6	0.6
18:0	7.5	3.3	18.4	4.0	25.3	4.7
18:1	62.9	31.2	75.7	39.6	87.4	44.3
18:2ω6	3.5	1.4	11.2	5.0	15.8	7.3
18:3ω3	1.5	0.9	1.7	1.0	2.1	0.7
20:1ω9	22.9	6.6	16.1	5.8	14.0	4.7
18:4ω3	7.5	2.3	5.0	2.1	4.0	1.6
20:4ω6	1.2	0.7	0.7	0.2	0.8	0.4
22:1ω11+ω13	21.9	4.0	14.6	2.3	11.6	2.1
20:4ω3	2.7	0.9	1.7	0.5	1.6	0.4
20:5ω3	21.6	5.3	14.4	4.0	11.6	3.1
22:5ω6	2.5	--	1.7	--	1.3	--
22:5ω3	4.0	1.5	2.7	1.5	2.1	0.3
22:6ω3	23.4	11.2	15.4	9.1	12.4	5.9

[a]May also contain 17:1.

salmon oil diet as described by Lee and Putnam (3). Six groups of fish, each group containing 50 fish, were then randomly selected. Each group was held in a 4-ft circular fiberglass tank. Water temperature was 11.5 C and the flow rate ca. 15 liters/min. Each of the three diets were fed to duplicate groups of fish three times daily. Food was offered as long as the fish continued active feeding. Mortality, feed consumption, and fish weight were recorded every 2 wk. The feeding experiment was terminated at the end of 14 wk. Five fish from each group were taken for fat content analysis on the whole fish. Methods for the extraction of total body lipid and further separation into phospholipids and preparation of methyl esters for gas liquid chromatography (GLC) analysis were described in a previous paper (1).

RESULTS AND DISCUSSION

Since the protein percentage and energy density of the three diets were identical, the growth response of the three dietary groups of fish was also approximately the same. Partial replacement of dietary herring oil by lard (diets 2,3) did not significantly change the fish weight gain and the feed efficiency in these two groups of fish (Table I). The accumulated mortality was low in all three diet groups of fish.

The FA composition of fish body lipid and phospholipids are shown in Table III. Total monoenoic acid content was highest in body lipid of diet 3-fish and lowest in diet 1-fish. The total $\omega 6$ and $\omega 3$ FAs in fish body lipid varied proportionally to the concentration of the respective FAs in the diets.

It is interesting to notice that the total saturated fatty acids (SFA) in fish body lipid remained fairly constant at ca. 24% regardless of the high SFA content in diets 2 and 3 (Tables II and III). Stickney and Andrews (9) reported similar results in their feeding experiments with catfish. A diet containing 10% tallow (41% SFA) was fed to a group of catfish, and another diet containing 10% menhaden oil (25% SFA) was fed to a second group of fish. After 10 wk at 20 C water temperature, the SFA content of the two groups of fish was found to be the same which was ca. 20% of the body lipid. It appears that a mechanism may exist in fish to regulate and maintain a proper level of body lipid saturation. Various publications have indicated that the degree of unsaturation of lipids in fish tissues was influenced by the environmental temperature (10-12).

The total amount of individual FA provided by dietary lipids and the quantity of each FA deposited in fish body is shown in Table IV. The portion of dietary FAs not retained by fish was metabolized and consumed for energy. A fairly high percentage of 16:0 and 18:0 was being metabolized in order to keep the body lipid saturation low. A large quantity of polyunsaturated and other FAs was also being metabolized. It was suggested that all FAs are subjected to metabolism except those FAs incorporated rapidly into phospholipids (13). It is shown in Table III that a high concentration of polyunsaturated FAs, especially 22:6$\omega 3$, was incorporated and retained in the phospholipids of the three dietary groups of fish. This appears to indicate the essentiality of 22:6$\omega 3$ in fish.

The results of this experiment supported the contention that the FA composition of body lipid reflected the FA composition of the diet for the following FAs: 14:0, 16:1, 18:2, 20:1, 18:4, 22:1, 20:4, 20:5 and 22:6 (Table II and III). However, there was no correlation between dietary lipid and fish body lipid contents of 16:0 and possibly 18:0. The 18:1 concentration in fish lipid was inversely proportional to the concentration of dietary polyunsaturated $\omega 6$ and $\omega 3$ FAs and directly proportional to the concentration of saturated FAs in the diet. These results are in good agreement with lipid metabolism in rat liver observed by Caster et al. (14).

It is evident that a partial replacement of herring oil by lard did not adversely affect fish growth and did not increase the saturation of the fish body lipid. The remaining dietary herring oil had provided sufficient quantity of $\omega 3$ FAs to satisfy the requirement of fish and to support normal trout growth.

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The Effect of Various Dietary Factors on the Size Distribution of Lymph Fat Particles in Rat

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ABSTRACT

By means of cellulose acetate electrophoresis and measurement of radioactivity, lipoproteins ($d \leqslant 1{,}006$) of lymph collected from rats fed a diet containing fats added to labeled fatty acids for 20-24 hr, have been separated into two kinds differing in their size. Fats with different saturated and unsaturated fatty acid content were tested: corn oil, lard, mutton tallow, tripalmitin, corn oil-lard mixture, and randomized palmitic-linoleic triglycerides. Factors studied were: (a) increased amounts of these fats given alone or included in a test meal; (b) added amounts of nutrients (other than fats) in the test meal; (c) adaptation to a diet containing 20% fat for 10 days. The major part of the labeled lymph lipid was always transported by the smaller particles. Size distribution of lymph fat particles was influenced by some of the factors studied. Generally, unsaturated fats produced higher amounts of larger particles.

INTRODUCTION

By means of cellulose acetate electrophoresis, we have shown (1,2), in mesenteric lymph collected from rats fed a diet containing ^{14}C or ^{3}H fatty acids, the presence of two labeled bands on the electrophoregrams: the first, M_o, remained at the origin whereas the second, $M_{\alpha 1}$, moved in the region of α_1-globulins (Fig. 1). Since these two kinds of lipoproteins ($d \leqslant 1{,}006$) can be separated by density gradient zonal centrifugation (2) and investigated by electron microscopy (2,3), we have found that the particles of band M_o have a larger diameter (0.1-0.3 μm) than those of band $M\alpha_1$ (diameter $\leqslant 0.1$ μM—mean value = 0.05 μm). The former could be similar to chylomicrons, the latter could be similar to very low density lipoproteins (VLDL) as described by Jones and Ockner (4). A small number of particles with diameters greater than 1 μm were sometimes found in zone M_o whatever the amounts of fat in the test meal, 80 or 800 mg (3). Previously we studied some effects of various dietary fats on the size and distribution of lymph fat particles in the rat (3), but these experiments performed with centrifugation and electron microscopic methods were rather time consuming whereas the electrophoretic method has the advantage of permitting a rapid study of various dietary factors on the size distribution of lymph fat particles, being a less laborious procedure than the others. In addition, this technique avoids the alteration of lymph. From our previous observations, it may be postulated that more zone M_o would be labeled, with higher relative content of larger particles in lymph.

We reported here the relative proportion of labeling of zone M_o compared to the total radioactivity in the 2 zones (M_o + $M\alpha_1$) obtained after electrophoresis of lymph collected from rats after feeding fats differing in nature (corn oil, lard, mutton tallow, tripalmitin, etc.), in amounts (80-1600 mg), in duration of administration (one meal or after 10 days of a diet containing 20% lipid). Sometimes fats were fed without adding other nutrients or the proportions of these nutrients in the test meal

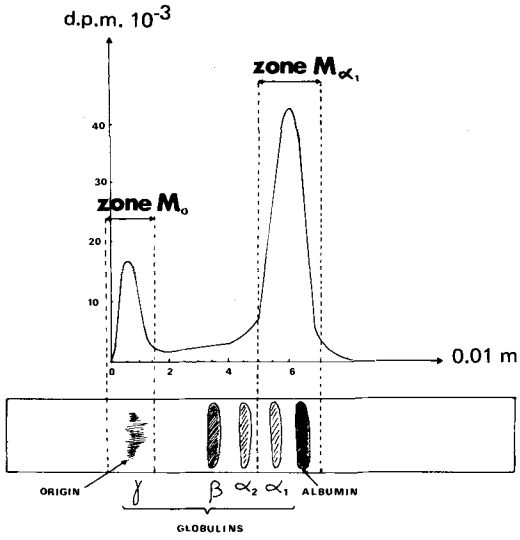

FIG. 1. Radiogram and cellulose acetate electrophoresis pattern of lymph of a rat fed corn oil labeled with 0.10 mCi 18:1 9-10^{3}H (Staining amidoschwarz).

TABLE I

Fatty Acid Composition of Natural Fats of Diets (in Moles)

Fats	Fatty acids						
	<16:0	16:0	16:1	18:0	18:1	18:2	>18:2
Corn oil		12.2	0.4	1.9	28.9	54.2	1.6
Lard	1.2	29.6	12.8	2.9	46.0	5.7	1.8
Mutton tallow	3.6	26.7	1.1	26.7	36.1	4.9	0.9

were modified.

MATERIAL AND PROCEDURES

Adult male Wistar rats, 200-250 g body weight, were fed either a commercial diet containing 17% protein and 4% lipid or a semi-purified diet containing 17% protein, 20% lipid for 10 days.

Cannulation of the main mesenteric lymph duct was performed. Sixteen hours later the animals received the test meal by stomach tube; chyle was collected during 20-24 hr and immediately subjected to cellulose acetate electrophoresis. This technique and measurement of radioactivity of zones M_o and $M\alpha_1$ on the electrophoregrams have been previously described (1,2).

All the electrophoretic analyses were performed with bands from the same batch (Cellogel, Chemetron, Milano).

Operated animals were allowed to drink ad libitum a solution of 0.7% NaCl, 0.2% KCl. Composition of the test meal always consisted of casein, sucrose, fat, 2 ml water, except in the experiments in which fats were fed alone; amounts of these nutrients will be indicated in each case. Fats tested were corn oil, lard, mutton tallow, tripalmitin synthesized with 9-10[^{3}H]-palmitic (100 μCi), randomized triglycerides synthesized with 9-10[^{3}H]-palmitic (100 μCi) and 1-[^{14}C]-linoleic (100 μCi) acids, a mixture of corn oil-lard (1:1 by weight). The fatty acid composition of the natural fats is indicated in Table I. The same fat was present in the high-fat diet and in the test meal in the adaptation experiments.

1-[^{14}C]- or 9-10[^{3}H]-palmitic, 9-10[^{3}H]-oleic, 1-[^{14}C]-linoleic acids (0.10 mCi-10 Ci/mM-CEA, Saclay, France) were added to the natural fats.

Examination of labeled fatty acids by thin layer chromatography (TLC) and gas liquid chromatography (GLC) indicated a radiopurity greater than 98%.

RESULTS

In lymph collected for 20-24 hr after the meal intake, the mass of lipids reached 200 ± 22 mg (mean ± SE, 19 animals) and 60 to 70% of the administered radioactivity was recovered in these lipids when the test meal contained 80-100 mg of fats. The proportions of mass and radioactivity of lipids recovered (35 to 50% of dose fed) were similar enough in each lymph sample collected for 20-24 hr when the test meal contained 400 mg of fats and more; individual variations were important; but generally the highest proportions were found as the amounts of fat fed were the lowest (400 mg) whatever the nature of dietary fat.

The major part of the radioactivity recovered (ca. 92%) was always present as triglycerides in total lymph and in zones M_o and $M\alpha_1$.

Effect of Meal (880 mg) Containing 80 mg of Various Fats Added to ^{3}H or ^{14}C Fatty Acid on the Percentages of Lipid Radioactivity Present in Zone M_o (Table II)

Some differences were observed among the samples of lymph according to the nature of the dietary fat. The proportion of larger fat particles (zone M_o) was higher after corn oil feeding than after other fats feeding; the smallest proportion of radioactivity of M_o was found with randomized triglycerides ingestion.

The nature of labelling seemed to cause no change in the percentage of M_o radioactivity.

Effects of Increasing Amounts of Fat Fed on the Percentages of Lipid Radioactivity Present in Zone M_o

In Table III are shown the proportions of radioactivity in zone M_o when the test meal contained either sucrose, casein and fat (A), or fat only (B).

In A, these values increased when the amounts of fat fed increased, but a limit value seemed to be reached after feeding 400 or 800 mg of fat.

Identical observations were made in B; the values remained relatively constant when the amounts of corn oil and lard fed increased (from 400 to 1600 mg). After 400 mg fat feeding, values were similar whatever the nature of fat (corn oil, lard, mutton tallow). Comparing results from A and B, no signifiant dif-

TABLE II

Percentages of Lipid Radioactivity Found in Zone M_0 by Comparison with the Total Radioactivity Present in Zones (M_0 + $M\alpha_1$) in Lymphs Collected from Rats Fed 80 mg Various Fats. Test Meal Contained in Addition 300 mg Casein, 500 mg Sucrose

Dietary fats				
Nature	Moles saturated FA[a] / Moles unsaturated FA	Tracers	Percentages of radioactivity in zone M_0 ^3H	^{14}C
Corn oil[b]	0.16	^3H 16:0 ^3H 18:1	12 ± 2 (2)[c] 12 ± 2 (8)	
Corn oil + Lard (1:1 by weight)	0.3	18:2 ^{14}C 16:0 ^3H	 10 ± 1 (2)	10 ± 1 (2)
Lard	0.5	^3H 16:0	8 ± 2 (3)	
Randomized triglycerides 18:2-16:0 1:1 by moles	0.5	^{14}C 18:2 ^3H 16:0	 6 ± 0.5 (2)	7 ± 1 (2)
Tripalmitin		^3H 16:0	9 ± 1 (2)	

[a]FA: fatty acids

[b]These values are not in agreement with our previous data (2) obtained under similar experimental conditions, but previously cellulose acetate strips came from different batches and the mesh size of gel may be a very important factor for retaining lymph larger fat particles.

[c]( ) = number of rats

TABLE III

Effect of Increasing Amounts of Fat Fed on the Percentages of Lipid Radioactivity Found in Zone M_0 by Comparison with the Total Radioactivity Present in Zones (M_0 + $M\alpha_1$) in Lymphs Collected for 20-24 hr

Dietary fats	Mass (mg) of fat fed				
	80	100	400	800	1600
Corn oil + ^3H 16:0	A[a] 12 ± 2[b] (2)[c] B[d]	13.8 ± 0.5 (2)	25 ± 3.4 (5) 26.4 ± 7.9 (4)	27.7 ± 6.9 (4) 22.9 ± 6.9 (2)	25 ± 3 (2)
Lard + ^3H 16:0	A 8 ± 2 (2) B	11 (1)	24.6 ± 8.2 (3) 21.4 ± 2.5 (3)	28.5 ± 10.2 (3) 25 (1)	26.6 ± 0.8 (2)
Mutton tallow + ^3H 16:0	A B		23.2 ± 1.8 (2) 13.8 ± 2.8 (2)		

[a]A: test meal always contained 300 mg casein, 500 mg sucrose, additional fats.

[b]The values represent the mean ± SE.

[c]( ) = number of rats

[d]B: test meal contained only fats.

ferences were observed in the percentages of radioactivity of M_0 as corn oil or lard was administered, but as mutton tallow was fed, the values were lower in B than in A.

Effect of Various Amounts of Nutrients in the Test Meal (Other than Fats) on the Percentages of Lipid Radioactivity of Zone M_0

The test meals always contained 400 mg of lard; for two rats, the meal contained 800 mg sucrose and 200 mg casein; on the other hand, for two other rats, the meal contained 800 mg casein and 200 mg sucrose.

No significant difference was found while the amounts of casein and sucrose were inverted in the test meal and the values (22 ± 3) are very close to these obtained in Table III (A) as amounts of casein and sucrose fed were different from those given in the present investigations.

Effect of Administration of a Diet Containing 20% Fat for 10 Days Before the Test Meal Intake on the Percentages of Lipid Radioactivity of Zone M_0 (Table IV)

Under these dietary conditions, the propor-

TABLE IV

Effect of Adaptation to a Diet Containing 20% Fat for 10 Days Preceding the Test Meal Intake on the Percentages of Lipid Radioactivity Found in Zone M_0 by Comparison with the Total Radioactivity Present in Zones (M_0 + $M\alpha_1$) in Lymphs[a]

Dietary fats	Tracers	Percentages of radioactivity zone M_0	
		^3H	^{14}C
Corn oil	^3H 16:0	32.4 ± 1.8[b] (6)[c]	
	^{14}C 18:2		30 ± 1 (2)
Lard	^3H 18:1	14.5 ± 1.4 (7)	
	^{14}C 16:0		15.5 ± 1.5 (2)
Mutton tallow	^3H 16:0	18.7 ± 2.8 (9)	

[a]Test meal always contained 600 mg casein, 1000 mg sucrose, 400 mg fats.

[b]The values represent the mean ± SE

[c]( ) = number of rats.

tions of radioactivity of zone M_0 were higher after corn oil feeding than after other fats and were the highest values found by comparison with those obtained previously (Table III) with the same dietary fat (nature and amount), but without adaptation to the high-fat diet. On the contrary, after adaptation to the diet containing 20% lard or tallow, the values of radioactivity of M_0 were unexpectedly lower than in the experiment in which only one meal with 400 mg fat was given. The decrease was more pronounced after adaptation to the diet containing lard.

DISCUSSION

By a combination of electrophoretic method and measurement of radioactivity, we have been able to investigate the effects of various dietary factors on the size distribution of lymph fat particles. Cellulose acetate gel may have a behavior somewhat similar to a sieve which prevents the movement of the larger particles (zone M_0) and we can conclude that the highest proportions of radioactivity of zone M_0 were due to the presence of highest amounts of chylomicrons with diameters ranging from 0.1 up to 0.3 μm as we established previously (3). However, under these experimental conditions, only exogenous fatty acids were taken into account although it is well established that endogenous fatty acids, which have a constant daily contribution in lymph (5-7) undergo the same process of digestion and absorption as do dietary fatty acids. Finally, exogenous and endogenous fatty acids would be incorporated into intestinal lipoprotein triglycerides.

In these studies, we confirm that the smaller lymph fat particles were always by far the most numerous (1-3). However, the size distribution of fat particles was influenced by the nature and the amounts of fat fed and these findings are in agreement with our previous results (3) and those of Fraser (8). After ingestion of only one test meal (Tables II and III), the effects of the nature of fats absorbed were more obvious with lower amounts of fat fed (80 or 100 mg) than with higher amounts (400 mg and more). The highest proportion of chylomicrons was found in intestinal lymph after 80 mg of unsaturated fat feeding (corn oil); as the molar ratio of saturated fatty acids/unsaturated fatty acids was $\geqslant 0.5$ in the dietary fat (tripalmitin, randomized triglycerides, lard), it seemed that the production of smaller fat particles was higher than that of chylomicrons. In this respect, our results are in accord with previous findings concerning the production of VLDL by intestine (9,10) or liver (11,12). Relationships between production of VLDL and saturation of absorbed or infused fatty acids could be a general occurence.

When amounts of fat administered increased, the proportions of lymph chylomicrons increased to a limit value (about 25% of the total fat radioactivity) whatever the nature of fat. When higher amounts of fat were absorbed (even if gastric emptying normally controls the pattern and rate of intestinal fat absorption), a greater length of small intestine may be required. As Wu et al. (13) and Sabesin et al. (14) have demonstrated that distal intestine provided larger fat particles than the proximal region in mucosal cells, these observations could explain our results; chylomicrons release in lymph could be the rate-limiting step for distal lymphatic fat transport (13). In the present investigation, different amounts of nutrients other than fats in the meal did not influence the size distribution of lymph fat particles; normally the amounts of intestinal

proteins may be sufficiently high to allow the synthesis of the protein moiety of these particles. The addition of proteins to the fats in the meal seemed to enhance the release of larger fat particles in lymph after mutton tallow feeding only (Table IIIA and B); we have no explanation to give for the results, so much the more as it is well known that the protein-fat ratio is lower in the larger particles than in the smaller ones.

In the lymph of animals on the high-fat diet for 10 days, the effects of saturated and unsaturated fats fed were more pronounced than after administration of only one test meal containing the same fats. However, these effects were not exactly in proportion to the saturated or unsaturated fatty acid content of fat fed, as was shown by the values obtained with mutton tallow or lard. No unequivocal explanation can be given for the results because very complex processes occurred; in particular, modification of enzymatic activities of pancreas and intestinal mucosa were found. Recently adaptative changes of the small intestine to increased dietary lipid have been described (15).

As a constant daily contribution of endogenous fatty acids in lymph lipids occurred in rats (5-7), in the present investigations this contribution was particularly obvious when the fat content of meal was low (80-100 mg)–because the amounts of lipid collected reached 200 ± 22 mg–and on the contrary, not easily seen when this content was higher (400 mg and more) because the endogenous fatty acid-exogenous fatty acid ratio in lymph lipids was very low. Radioactivity determinations were always performed with accuracy, but mass determinations were not always reliable as the values were very low, for instance those of lipids of M_0 in lymph collected for 20-24 hr after ingestion of meals containing 80 or 100 mg of fats; in these cases, it was impossible to determine the distribution of endogenous fatty acids between zones M_0 and $M\alpha_1$. Let us notice that under these dietary conditions, we have found (16) a higher proportion of endogenous fatty acids in zone M_0 than in zone $M\alpha_1$, at the absorption peak as the mass of M_0 lipids was the highest. When the amounts of fat fed were 400 mg and more (Table III and IV), it is easy to determine the mass of M_0 with accuracy; the specific activity of fatty acids determined in each zone, M_0 and $M\alpha_1$, was similar enough for each sample of lymph. Under these conditions, we can conclude that the distribution of endogenous fatty acids among zones M_0 and $M\alpha_1$ was proportional to their exogenous fatty acid content.

Similar proportions of labeling in chylomicrons and smaller fat particles were found after administration of the same test meal, single or doubly labeled with saturated or (and) unsaturated fatty acid. This observation emphasized that these labeled fatty acids were esterified into triglycerides and that these components were distributed at a constant rate, between the two kinds of particles after administration of the same test meal.

As the production of higher amounts of larger fat particles was found after unsaturated fat feeding and as the removal of these particles was faster than that of smaller ones (17), these observations may explain the lipid-lowering effect of unsaturated fats in blood.

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Purification of Surfactant from Lung Washings and Washings Contaminated with Blood Constituents

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ABSTRACT

A rapid and simple method capable of purifying surfactant from rabbit alveolar washings and from washings contaminated with serum has been developed. The sample, containing 16% NaBr, is placed beneath a two-layer discontinuous gradient of NaBr. After centrifugation, the surfactant is found near the top of the gradient tube at a density of 1.085 at 4 C while the contaminating material remains near the bottom. The lipid composition of surfactant from lung washings of normal animals isolated by this method compares quite favorably with surfactant isolated by much more elaborate and time consuming methods. Surfactant purified from mixtures of ^{3}H-palmitate labeled rabbit serum and lung washings (1.6 mg serum phospholipid:1 mg washing phospholipid) contained less than 3% of the phospholipid radioactivity. The phospholipid composition of this band was quite similar to that of surfactant from normal lung washings, but the protein content was much higher. A second density gradient centrifugation removed 90% of this protein resulting in a surfactant fraction with a phospholipid to protein ratio similar to that of surfactant from normal lung washings. These findings demonstrate that this purification method is capable of removing a large proportion of both serum phospholipids and proteins from lung washings contaminated with serum, making this method uniquely suitable for evaluation of surfactant in pathologic conditions of the lung.

INTRODUCTION

A major problem in the study of lung surfactant, particularly in pathologic conditions, is the lack of appropriate methods to separate the surfactant from blood components transudated into the air spaces. Current methods for the isolation and purification of lung surfactant (1-7) are not applicable to contaminated material because early in the procedure differential centrifugation is used. Recently we have reported that sedimentation results in the entrapment of protein within aggregates of surfactant liposomes, with the amount of protein in the sediment being directly dependent on the amount of protein in the original sample (8). These aggregates are not readily dispersed; therefore, density gradient centrifugation of the entire noncellular washing appears to be a better procedure than purifying the sedimented surfactant fraction on density gradients.

This paper describes a relatively simple method of density gradient centrifugation for purifying lung surfactant from both clean lung washings and contaminated samples. It differs from other methods in that no sedimentation step is used and also that the sample is placed beneath the gradient instead of being spread throughout the tube or on top of the gradient.

EXPERIMENTAL PROCEDURES

Lung washings were obtained from New

TABLE I

Density Gradients for Isolation of Lung Surfactant

Rotor and tube (Spinco)	SW25.2	SW27.1	SW50L
Dimensions of tube	1¼ x 3½ in.	5/8 x 4 in.	½ x 2 in.
Total volume of gradient	60 ml	17 ml	5 ml
Gradients layers			
No NaBr	8 ml	4 ml	1 ml
13% NaBr	32 ml	8 ml	3 ml
Sample in 16% NaBr	20 ml	5 ml	1 ml
Centrifugaiotn			
g max	90,000	116,000	98,000
time	3 hr	2 hr	1 hr

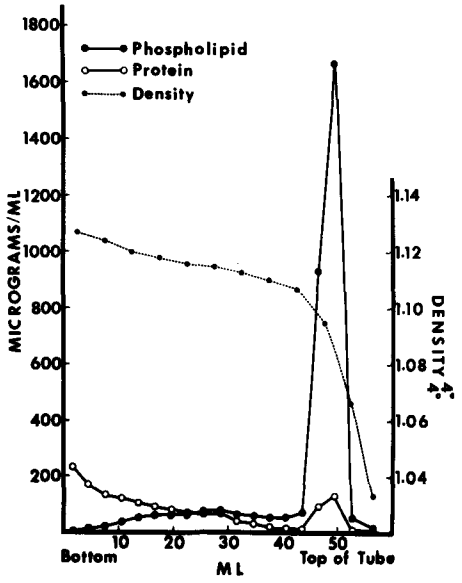

FIG. 1. Distribution of phospholipid and protein following density gradient centrifugation of noncellular lung washing at 4 C in SW 25.2. The phospholipid band is centered at density of about 1.085 and contains a small amount of protein.

Zealand white male rabbits weighing 1.4 to 1.6 kg, by two consecutive alveolar lavages with 70 ml of 0.9% NaCl warmed to 37 C before use. The lung washings were centrifuged for 5 min at 480 *g* to remove the cells and then used to prepare gradients for purification of the surface active material or frozen at -70 C for future use. Serum was obtained from a second group of rabbits 3 hr after an intravenous injection of 600 μCi of ^{3}H-palmitate. The serum from these rabbits was pooled and the specific activity of the phospholipid was 2.4 x 10^4 dpm/mg phospholipid.

Discontinuous density gradients were prepared at 4 C in three different sizes of Spinco centrifuge tubes (Table I). NaBr was added to the noncellular lung washings to give a concentration of 16% (density = 1.14). The lung washings containing 16% NaBr were placed on the bottom of a tube, over-layered with 13% NaBr (density = 1.11) in 0.9% NaCl and then with saline containing no NaBr. In separate experiments, the ability of the above purification method to separate surfactant from serum components was tested by adding ^{3}H-palmitate labeled serum to noncellular lung washings in a volume ratio of 1:3, prior to gradient preparation.

The gradients were centrifuged in a Spinco model L5-50 ultracentrifuge for 1-3 hr (Table I) at 4 C and stopped without use of the brake. Centrifugations for longer periods of time, up to 18 hr, were tried with the SW25.2 rotor and found to be unnecessary and actually undesirable since they resulted in a less steep gradient in the part of the tube where surfactant was found yielding a wider and more diffuse surfactant band. Following centrifugation, fractions were collected from the bottoms of the tubes after puncturing with a needle held in a plastic cylinder. In some experiments, the gradient fractions containing surfactant were further purified on a second gradient. The NaBr content of the surfactant fractions was adjusted to 16% and using this solution as bottom layer, new gradients were prepared and centrifuged in the same manner as above. Density measurements were made by pooling corresponding fractions from several gradients and weighing in a precooled 10 ml pycnometer.

Surface tension was measured in a modified Langmiur-Wilhelmy surface balance (9) essentially as described by King and Clements (5). Aqueous suspensions containing 20μg of phospholipid were mixed with 2 volumes of isopropyl alcohol and applied to a subphase of 0.9% NaCl at 24 C. Samples were considered surface active if they lowered the surface tension to less than 12 dynes/cm (1).

The protein concentrations of the samples were measured by the method of Lowrey et al. (10), using sodium dodecyl sulfate to solubilize the particulate material present in the fractions containing high concentrations of lipids.

Lipids were extracted using chloroform-methanol, 2:1 (11). Phospholipids were separated from neutral lipids by thin layer chromatography (TLC) on 250 micron thick Silica Gel G plates with petroleum ether, ether, and acetic acid (80:20:1). The phospholipid spot was scraped into a conical centrifuge tube and the phospholipids were eluted from the gel by washing the gel once with chloroform-methanol (2:1) and then with methanol. Aliquots of the phospholipid fractions were used to determine the concentration of the phospholipid (12) and for counting the radioactivity in a liquid scintillation counter.

The phospholipids were separated by two-dimensional chromatography on silica gel-impregnated paper (12). For determination of the phosphorus content of each spot, the papers were stained with Rhodamine 6G and the amounts of individual phospholipids present, as well as total phosphlipid content of the various samples, were determined by measuring the phosphorus content after digestion of the lipids with perchloric acid (12). The amount of phospholipid present was calculated on the assumption that the phospholipids contained an

TABLE II

Distribution of Phospholipids

	Percent of total phospholipids[a]					
	LPC	Sph	PC	PI	PE	PG
Noncellular lung washings	ND[b]	ND	87.4 (1.7)[c]	2.4 (0.6)	2.5 (0.6)	7.7 (1.5)
Surfactant from lung washings	ND	ND	85.9 (1.1)	2.5 (0.2)	3.1 (0.6)	8.5 (0.7)
Serum-lung washing mixture (1:3)	11.1 (2.2)	2.9 (1.1)	72.8 (3.0)	5.1 (1.7)	5.0 (0.6)	3.1 (0.6)
Surfactant from serum-lung washing mixture – 1 gradient	0.8 (0.3)	1.1 (0.6)	81.3 (1.3)	3.7 (0.5)	5.0 (1.2)	8.1 (0.5)
Surfactant from serum-lung washing mixture – 2 gradients	0.6 (0.3)	0.7 (0.4)	84.7 (1.2)	2.6 (0.4)	2.8 (0.6)	8.6 (1.4)

[a]LPC = lysophosphatidyl choline, Sph = sphingomyelin, PC = phosphatidyl choline, PI = phosphatidyl inositol, PE = phosphatidyl ethanolamine, PG = phosphatidyl glycerol.

[b]ND = none detected.

[c]Numbers in parentheses indicate standard error.

average of 4% phosphorus.

For determination of the fatty acid composition of the phosphatidyl choline (PC) lipids were separated by 2-dimensional TLC on Silica Gel G plates with chloroform-methanol-ammonium hydroxide (140:50:7) in the first direction and chloroform-methanol-acetone-acetic acid-water (100:20:40:20:10) in the second direction. After development, the plates were dried and the lipid spots detected under ultraviolet light after spraying with 0.01% Rhodamine 6G in water. The PC spots were scraped into tubes and the methyl ester derivatives of the acyl groups were prepared by alkaline methanolysis (13). The fatty acid methyl esters were separated by gas liquid chromatography using a Varian model 2100 with 2 mm x 6 ft column packed with 10% EGSS-X on 100/120 mesh Gas Chrom P. The column was standardized using the National Institutes of Health fatty acid methyl ester standards (Applied Science Laboratories, State College, PA). The fatty acids were identified by comparison of the relative retention times with standards and by interpolation from a semilog plot of the carbon number of the free acid versus the logarithm of its respective retention time.

RESULTS

Isolation of Surfactant from Lung Washings

Following centrifugation of density gradients prepared with lung washings, a band of white particulate material was readily observed near the top of the gradient tube at a density of 1.085 at 4 C. Fractions collected from these gradient tubes were analyzed for phospholipid and protein content and the distribution of phospholipid and protein from one such gradient is shown in Figure 1. The fractions corresponding to the visible band of material contained 80% (± 3.5 S.E.) of the phospholipid but only 12% (± 1.5) of the protein present in the gradient. Recentrifugation of this surfactant on a second gradient resulted in recovery of more than 95% of both the phospholipid and protein in the surfactant band. Both the noncellular lung washings used to prepare the gradients and the gradient fraction from the phospholipid-rich band were surface active with the purified surfactant lowering the surface tension to 5 dynes/cm. Other fractions from the density gradient tubes showed no surface activity indicating that the lung surfactant was concentrated in the narrow visible band.

Phospholipid analysis revealed no significant differences between the phospholipid composition of the washings and of the surfactant band (Table II). Both the washings and the surfactant band contained more than 85% PC. A significant amount of phosphatidyl glycerol (PG) and smaller quantities of phosphatidyl ethanolamine (PE) and phosphatidyl inositol (PI) were also present. The fatty acid composition of the PC from the lung washings and the surfactant purified from the washings differed only slightly (Table III). The surfactant band contained slightly more palmitic acid (16:0) and less 18-carbon fatty acids than did the original washings.

Isolation of Surfactant from Serum-Lung Washing Mixtures

In order to determine if this simple method of surfactant purification was capable of sepa-

TABLE III

Major Fatty Acids of Phosphatidyl Choline

	Percent of total fatty acids							
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4
Noncellular lung washings	2.2 (0.2)[a]	62.3 (1.8)	5.8 (0.3)	2.1 (0.2)	18.0 (1.3)	8.6 (0.7)	1.0 (0.1)	trace
Surfactant from lung washings	2.7 (0.5)	67.8 (1.7)	5.6 (0.3)	1.7 (0.2)	14.4 (1.1)	6.9 (0.9)	0.9 (0.1)	trace
Serum-lung washing mixture (1:3)	1.4 (0.6)	37.4 (1.1)	2.7 (0.2)	11.5 (0.9)	19.3 (0.7)	23.6 (0.2)	2.1 (0.2)	2.0 (0.4)
Surfactant from serum-lung washing mixture-1 gradient	1.5 (0.3)	60.2 (0.2)	5.2 (0.3)	3.2 (0.1)	17.9 (0.3)	10.6 (0.5)	1.1 (0.1)	0.3 (0.1)
Surfactant from serum-lung washing mixture-2 gradients	1.4 (0.3)	60.9 (0.3)	4.4 (0.2)	2.7 (0.2)	19.5 (0.3)	9.9 (0.2)	1.2 (0.1)	trace

[a]Numbers in parentheses indicate standard error

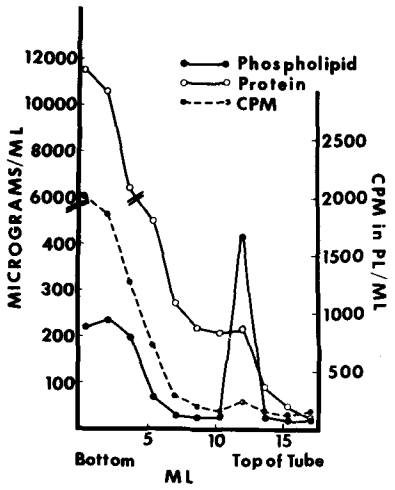

FIG. 2. Distribution of phospholipid and protein and radioactivity following density gradient centrifugation of serum-lung washing mixture (1:3) in SW27.1. Although less than 1% of the protein in the gradient tube is found in the surfactant band, these fractions remain contaminated with plasma proteins. Less than 3% of the phospholipid radioactivity is in the fraction containing surfactant.

rating surfactant from serum proteins and lipids often found in lung washings, especially in various pathological conditions, lung washings and ^{3}H-palmitate labeled serum were mixed prior to density gradient centrifugation. Of the total phospholipid in the mixture, 60% originated from serum and 40% from the lung washings. The distribution of phospholipid and protein in a 17 ml gradient following centrifugation of such a mixture is shown in Figure 2. A particulate band, rich in phospholipids, was found in the same position as was the surfactant from lung washings only. However, only 37% (± 4) of the total phospholipid from the mixture was in the surfactant band compared with about 80% from lung washings only. In addition, less than 3% of the phospholipid radioactivity was found in the surfactant band (Fig. 2). Although less than 1% of total protein in the gradient was present in the surfactant fraction, the very large amount of protein found in serum resulted in a considerable contamination of the surfactant band with protein. The fact that the density at which the band was located was the same as for washing only, indicated that the excess protein was not associated with the surfactant phospholipid. This was also shown by using a part of the surfactant band to prepare a second gradient. After centrifugation of the second gradient, only a small amount of protein remained with the phosphlipid band (Fig. 3). Eight percent of the protein and 87% of the phospholipid of the gradient were in this band. It may be noted, however, that almost all of the phospholipid radioactivity in this gradient is found in the surfactant band, indicating that the small percent of serum phospholipids that do become associated with the surfactant are not removed by a second density gradient centrifugation.

The various samples and mixtures used in these experiments were tested for surface activity. Following mixing with serum, the cell-free lung washings were not surface active. The surfactant fractions from both the first and second gradients of the lung washing-serum mixture were surface active lowering the surface tension to 10 and 8 dynes/cm, respectively.

Phospholipid analysis showed that serum-washing mixtures contained more lysophosphatidyl choline (LPC), sphingomyelin (Sph), PI and PE and less PC and PG than the lung washings (Table II). The surfactant from both the first and second gradients contained less of the serum components, LPC, Sph, PI, and PE, and more of the surfactant phospholipids, PC

and PG, with the final surfactant band having a phospholipid composition quite similar to that of the surfactant from the lung washings only.

The same pattern of changes can be seen in the fatty acid composition of the PC (Table III), with the serum-washing mixture containing less palmitic and more stearic (18:0), linoleic (18:2), and arachidonic (20:4) acids than the lung washings. Gradient centrifugation resulted in the purification of a surfactant band which contained more palmitic and less stearic, linoleic and arachidonic acids than the original mixture. Although not identical, this fatty acid distribution closely resembled that of surfactant from lung washings.

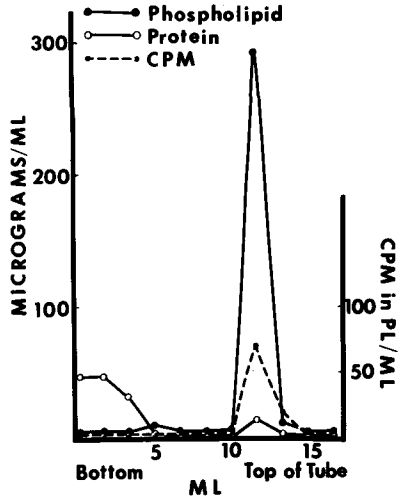

FIG. 3. Distribution of phospholipid and protein following a second density gradient centrifugation of surfactant band obtained from serum-lung washing mixture. A large portion of contaminating protein has been separated from the surfactant band.

DISCUSSION

This paper introduces a simple and rapid method for the purification of surfactant from alveolar washings and from washings contaminated with serum. Surfactant has a density of about 1.085 at 4 C, which is lower than that of most of the contaminating components of lung washings or serum. Therefore, when the mixture containing surfactant is placed beneath a gradient of appropriate densities, the surfactant moves upward into the gradient while the other components, such as protein (densities of 1.3 or greater) and membrane fragments and cell organelles (densities greater than 1.1) would be expected to move downward. In practice, there is little tendency for at least the smaller components to sediment, and when present in very large concentrations, the small components diffuse upward. However, placement of the sample beneath the gradient does avoid the movement of the contaminating materials through the gradient as happens when the sample is placed on the top or spread throughout the tube at the start of centrifugation. Surfactant components are quite large and move rapidly to their isopycnic density. Use of three different sizes of gradients, all of which gave similar separations, makes this method quite adaptable to samples from various sources. SW25.2 gradients with 20 ml of sample are useful for purification of surfactant from large quantities of lung washing, while the 5 ml gradients, using 1 ml of sample, are preferable when only small volumes are available such as tracheal aspirates from infants or amniotic fluids from experimental animals.

The surfactant isolated from lung washings by the method described in this report compared quite favorably with the surfactant isolated by more elaborate schemes (4,6,14). The surfactant is found at a density of 1.085 in close agreement with the reports of King and Clements (5). The major component is PC containing saturated fatty acids. The second most abundant phospholipid is PG, a finding which is in agreement with recent reports (15,16). Although we did not quantitate the amounts of neutral lipids present, TLC revealed that cholesterol was the most abundant neutral lipid, with small amounts of triglycerides and free fatty acids also present, a finding also in agreement with previous reports (3,4,6,14). The amount of protein in the surfactant fraction of lung washings was lower than that reported by most other investigators (2,4-6). This lower protein content was probably due to the fact that sedimentation was not used before density gradient centrifugation (8).

The method presented here is also capable of separating surfactant from large amounts of contaminating protein and nonsurfactant phospholipids as demonstrated by the recovery of a surfactant band from gradients prepared with serum-lung washing mixtures. The phospholipid composition of this surfactant was essentially identical to that of surfactant from lung washings only, while the fatty acids of PC from the two sources differed more, suggesting that the major phospholipid contaminant from the serum is PC. The amount of this contamination is quite small, as indicated by recovery of less than 3% of the phospholipid radioactivity in the surfactant band, while most of the serum phospholipids remained near the bottom of the gradient. The large excess of serum proteins was also removed from the surfactant band fol-

lowing two centrifugations.

Currently used methods for surfactant purification generally involve several successive periods of centrifugation and rather large amounts of lung tissue or washings as starting material and, therefore, are not applicable to large numbers of samples or to samples where the amount of available tissue or washings is limited. In addition, these methods have seldom been applied to any samples other than those from healthy animals, presumably because they are not adequate to separate surfactant from large amounts of contaminating material found in various pathological conditions. They likewise have not been widely applied to human material, since samples recovered at autopsy are also contaminated with blood. The method of purification introduced in this study has been successfully applied to normal and edematous human lung washings, amnitotic fluid samples, pharyngeal aspirates from newborn infants, and tracheal aspirates collected routinely from infants on respirators (18). Use of this method permits clinical evaluation of maturation and pathological changes in the functional components of the surfactant system.

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The Phylogenetic Distribution of Sterols in Tracheophytes

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ABSTRACT

The sterols of nine mature plant species in seven families ranging from the subphylum Lycopsida through the Filicopsida and the classes Gymnospermae and Angiospermae in the Pteropsida were structurally and stereochemically defined. Two plant categories were found. In the first, comprised by *Dryopteris (Thelypteris) noveboracensis, Polystichum acrostichoides, Dennstaedtia punctilobula, Osmunda cinnamomea, Ginkgo biloba, Cucurbita pepo,* and *Kalmia latifolia,* 24α-alkylsterols were dominant and were composed principally of 24α-ethylcholesterol (sitosterol) or (in *Cucurbita pepo*) 24α-ethyllathosterol and its *trans*-22-dehydro derivative (spinasterol). Depending on the species, small amounts of 24α-ethyl-*trans*-22-dehydrocholesterol (stigmasterol), 24α-methylcholesterol (campesterol), 24β-methylcholesterol (dihydrobrassicasterol, always less than campesterol), cholesterol, lathosterol, 24α-ethyllathosterol, 24ξ-methyllathosterol, *trans*-24-ethylidenelathosterol (Δ^7-avenasterol), and (tentatively identified) 24-ethyl-24(25)-dehydrolathosterol were present. *Spinacea oleracea* was also confirmed as belonging to Category I, and, except as described in what follows, Category I represents all other configurationally investigated vascular plants. The second category of plants contained only 24β-ethylsterols. Only one species (*Kalanchoe daigremontiana*) belonging to the family Crassulaceae was found, but one other (the genus *Clerodendrum* in the family Verbenaceae) is already known. *K. daigremontiana* contained 25(27)-dehydroclionasterol (clerosterol) and 25(27)-dehydroporiferasterol. The primitive *Lycopodium complanatum* was intermediate between Categories I and II; the sterols with a 24-C_2-group had only the 24α-configuration (sitosterol with some stigmasterol), but the principal sterols with a 24-C_1-group (ergosterol and dihydrobrassicasterol) possessed the 24β-configuration. *C. pepo* seeds, which are already known to contain principally 24β-ethylsterols, contrast sharply with our finding that tissue (pericarp of the fruit) from the mature plant contains only 24α-ethylsterols. This apparent evolutionary recapitulation (Category II to I) during development coupled with statistical dominance of Category II plants among the algae and fungi and of Category I plants in the Tracheophytes, and the existence of an intermediate type in the species examined from the lower Tracheophyte (Lycopsida) lead logically to the conclusion that the 24α-alkyl structure, especially 24α-ethyl-Δ^5-sterols (sitosterol and the much rarer stigmasterol) constitutes the most highly evolved type of 24-alkylsterol. By inference from our knowledge of biosynthesis, corroborated by the spectrum of sterols found here, the pathway [through $\Delta^{24(25)}$-sterols] leading to 24α-alkylsterols appears to be higher than the pathway [through $\Delta^{25(27)}$-sterols] which leads to 24β-alkylsterols. The sterols of these and other plants were also found amenable to classification according to their nuclear unsaturation (Δ^5, A; Δ^7, B; and $\Delta^{5,7}$, C). Tracheophytes of Category A have been most frequently encountered, but *C. pepo* was shown to be of Category B throughout its ontogeny. While no Tracheophytes of pure Category C have been discovered, *L. complanatum* was shown to be of the mixed A-C-Type. Based on these facts and ideas, some previously suggested lines of botanical evolution are examined. The chemical data fail to verify a line from Magnoliales to the *Cucurbitaceae,* from Magnoliales through Theales to Ericales, nor from Ranales to Saxifragales. However, they are consonant with a relationship between Cucurbitaceae and Theales and between Rosales and Lamiales. Triterpenoids found in various of the families studied included cycloartenol and friedelin. The spectroscopic properties of the latter are described.

INTRODUCTION

With the recent development of reversed

phase chromatographic methods for the separation of homologous sterols (1-3) and of proton magnetic resonance spectroscopy at 220 MHz for configurational analysis at C-25 (4,5), it has become possible to examine the relationship between structure and occurrence of sterols with quantitative and stereochemical precision and thereby to shed light on the biosynthetic pathways operating at various stages of the evolutionary hierarchy. Proton magnetic resonance (PMR) spectroscopy is of special significance. Since we have not seen the same spectrum given by two different sterols, the spectra at 220 MHz constitute a "finger print." In the case of the Phylum Tracheophyta, we recently used this methodology to examine the sterols of representatives drawn from a cross-section of ferns and seed-bearing plants (4). One plant each in the subphylum Filicopsida (the New York fern) and in the Pteropsida class Gymnospermae (a pine) were examined. Among the Angiospermae, we included the two most primitive orders (Magnoliales and Ranales), one more advanced order (Leguminales) of the woody Dicotyledons (Lignosae), and one order (Cruciales) in the herbaceous Dicotyledons. In all cases, 24α-ethylcholesterol was the major component and no 24β-ethylsterol was detectable. However, contrary to earlier suppositions, the 24-methylcholesterol was a mixture of 24α- and 24β-methyl epimers in which the former was dominant. This work made it clear that two pathways must operate simultaneously, both generally as well as in a given plant, at the 24-methyl level. On the other hand, at the 24-ethyl level, a pathway usually exists apparently only for a single configuration (24α), but the problem is complicated by the fact that 24β-ethylsterols do exist in higher Tracheophytes. They have been found in the seeds of several genera in the family Cucurbitaceae of the order Cucurbitales (6-9) and in two species of the genus *Clerodendrum* in the order Verbenales (10,11). In order to ascertain just how common one or other of the configurations is, we have continued our investigation of plants arranged phylogenetically.

In the present study, we chose a representative (*Lycopodium complanatum*) of the subphylum Lycopsida which is less advanced than either the Filicopsida or Pteropsida previously analyzed. We also chose three more ferns [Hay-scented and Christmas ferns in the common family Polypodiaceae and Cinnamon in the family of so-called "flowering ferns" (Osmundaceae)] as well as a representative (the maidenhair tree) of the Gymnospermae which is less advanced than the pine previously studied and a representative (*Kalanchoe daigremontiana*) of the biochemically and morphologically primitive Crassulaceae family in the herbaceous angiosperm order Saxifragales. Furthermore, the New York fern (4) was reexamined for minor components detected by gas liquid chromatography (GLC) but not previously reported on. From the woody Dicotyledons, we also chose a plant (mountain laurel) in the family Ericaceae from the order Ericales which is believed (12) to have arisen from Magnoliales through an order (Theales) different from those (Dilleniales, Coriariales, and Rosales) suggested (12) as intermediates to the previously studied (4) Leguminales. Finally, we examined the fruit and leaves of the pumpkin from the family Cucurbitaceae in the order Cucurbitales, because the seeds are known (6-9) to contain principally 24β-ethylsterols.

The nomenclature used in this paper takes cholesterol, its Δ^7-analog, lathosterol, and certain other sterols with well established trivial names as parents. It is summarized in Table I.

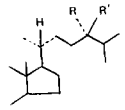
MATERIALS AND METHODS

Cultivated male maidenhair tree leaves (*Ginkgo biloba*), and the leaves and pericarp (fleshy part of the fruit) of cultivated pumpkin plants (*Cucurbita pepo*), the leaves of wild mountain laurel (*Kalmia latifolia*), and fronds of the wild New York fern (*Dryopteris noveboracensis*) (also known as *Thelypteris noveboracensis*) were collected in southeastern Pennsylvania. Specimens of *Kalanchoe daigremontiana* Hamet and Perr., native to S.W. Madagascar, were grown in the Drexel University greenhouse. Whole plants were used for chemical study. One sample (stems and fronds) of *Lycopodium complanatum* and the sample of the Christmas fern (*Polystichum acrostichoides*) (fronds) were collected from wild growths in Massachusetts in the summer. The other sample of *L. complanatum* was collected in Virginia in November. The fronds of the hay-scented [*Dennstaedtia punctilobula* (Michx.) Moore] and Cinnamon (*Osmunda cinnamomea* L.) ferns were collected from wild growths in Maryland. For *Spinacea oleracea*, we used fresh leaves sold commercially as food.

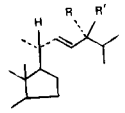
Except as noted, the 4,4-dimethyl- and 4-desmethyl substances were isolated from the specimens as previously described (4) through continuous acetone extraction, saponification, and sequential chromatography on alumina with ether graded into hexane and on lipophilic Sephadex (Lipidex-5000) with 5% hexane in methanol. This procedure yields sterols from both the free and esterified pools. Spectral and gas liquid chromatographic procedures were

TABLE I

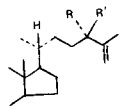
Sterol Nomenclature Used



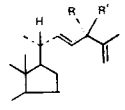
Substitution		Name	
R	R′	Δ^5-Series	Δ^7-Series
H	H	Cholesterol	Lathosterol
H	CH_3	24α-Methylcholesterol (Campesterol)	24α-Methyllathosterol (24-Epifungisterol)
CH_3	H	24β-Methylcholesterol (22-Dihydrobrassicasterol)	24β-Methyllathosterol (Fungisterol)
H	C_2H_5	24α-Ethylcholesterol (Sitosterol)	24α-Ethyllathosterol (22-Dihydrospinasterol)
C_2H_5	H	24β-Ethylcholesterol (Clionasterol)	24β-Ethyllathosterol (22-Dihydrochondrillasterol)



Substitution		Name	
H	H	*trans*-22-Dehydrocholesterol	*trans*-22-Dehydrolathosterol
H	CH_3	*trans*-22-Dehydrocampesterol (24-Epibrassicasterol)	24α-Methyl-*trans*-22-dehydrolathosterol (22-Dehydro-24-epifungisterol)
CH_3	H	Brassicasterol	24β-Methyl-*trans*-22-dehydrolathosterol (*trans*-22-Dehydrofungisterol)
H	C_2H_5	*trans*-22-Dehydrositosterol (Stigmasterol)	24α-Ethyl-*trans*-22-dehydrolathosterol (Spinasterol)
C_2H_5	H	*trans*-22-Dehydroclionasterol (Poriferasterol)	24β-Ethyl-*trans*-22-dehydrolathosterol (Chondrillasterol)



Substitution		Name	
CH_3	H	24β-Methyl-25(27)-dehydrocholesterol (Codisterol)	24β-Methyl-25(27)-dehydrolathosterol
C_2H_5	H	24β-Ethyl-25(27)-dehydrocholesterol (Clerosterol) (25(27)-Dehydroclionasterol)	24β-Ethyl-25(27)-dehydrolathosterol



Substitution		Name	
CH_3	H	24β-Methyl-*trans*-22,25(27)-bisdehydrocholesterol (*trans*-22-Dehydrocodisterol)	24β-Methyl-*trans*-22,25(27)-bisdehydrolathosterol (*trans*-22,25(27)-Bisdehydrofungisterol)
C_2H_5	H	24β-Ethyl-*trans*-22,25(27)-bisdehydrocholesterol (25(27)-Dehydroporiferasterol) (*trans*-22-Dehydroclerosterol)	24β-Ethyl-*trans*-22,25(27)-bisdehydrolathosterol (25(27)-Dehydrochondrillasterol)

also described earlier (4). PMR spectra were performed at 220 MHz in $CDCl_3$ unless otherwise noted. Retention times in GLC are described (RRT) in terms of their values relative to the retention time of cholesterol, and the liquid phase was XE-60 at 235 C. Representative mass and PMR spectra are listed in Tables II–VI for sterols derived from plants examined in the present work. For comparison, especially in the case of epimers at C-24, authentic sterols are included for some of which spectral data have not heretofore been published. The corresponding information in the one case of a mature plant in which 24β-ethylsterols were found to be dominant is given in that part of the Results section dealing with *K. daigremontiana.* Additional data on Δ^7-sterols is given in the Results section under *C. pepo.*

RESULTS

Lycopodium complanatum

The mass spectrum of the 4-desmethylsterol fraction from alumina of the specimen collected in the summer showed the presence of materials with molecular weights of 396, 414, 400, and 412 (in descending order of the intensity of M^+). Three sterol fractions [50 mg combined from 118 g (wet wt) of plant] were then separated on Sephadex. The fastest

TABLE II

Typical Mass Spectra of Δ^5 and Δ^7-Sterols from Tracheophytes

	m/e, %						
	Cholesterol series				Lathosterol series		
Side chain	24–H	24–CH_3	24–C_2H_5	24–C_2H_5-Δ^{22}	24–H	24–C_2H_5	24–C_2H_5–Δ^{22}
Source	Christmas Fern	Christmas Fern	Christmas Fern	Soybeans	NY Fern	NY Fern	*C. pepo* Pericarp
Fragmentation							
M^+	386, 100	400, 100	414, 100	412, 66	386, 100	414, 100	412, 28
M^+-CH_3	371, 32	385, 29	399, 29	397, 9	371, 29	399, 26	397, 10
M^+-H_2O	368, 47	382, 42	396, 44	394, 11	368, 2	369, 2	394, 1
$M^+-CH_3-H_2O$	353, 33	367, 30	381, 28	379, 13	353, 5	381, 4	379, 1
$M^+-C_3H_7$	-	-	-	369, 22	-	-	369, 13
M^+-85	301, 43	315, 41	329, 39	327, 5	-	-	-
$M^+-C_7H_{11}$ or $_{12}O$	275, 56	289, 47	303, 44	300, 42	-	-	-
M^+–(SC = Side Chain)	273, 26	273, 241	273, 25	273, 23	273, 21	273, 21	273, 31
M^+–SC–2H	271, 15	271, 2	271, 2	271, 59	-	-	271, 100
M^+–SC–H_2O	255, 36	255, 29	255, 28	255, 100	255, 75	255, 67	255, 40
M^+–SC–H_2O–2H	253, 6	253, 1	-	-	-	-	-
M^+–SC–C_3H_6	231, 30	231, 27	231, 24	231, 18	231, 31	231, 25	231, 18
M^+–SC–C_3H_8	229, 18	229, 12	229, 11	229, 21	229, 24	229, 20	229, 21
M^+–SC–C_3H_6–H_2O	213, 43	213, 39	213, 39	213, 45	213, 33	213, 24	213, 20
M^+–SC–C_3H_8–H_2O	211, 9	211, 2	211, 1	-	211, 2	211, 1	211, 3

TABLE III

Mass Spectra of Tracheophyte Ergosterol and Related Standards

Side chain	m/e, % in 7-Dehydrocholesterol series					
	24–H[a]	24α–CH_3[b]	24α–CH_3–Δ^{22}[c]	24β–CH_3–Δ^{22}[d]	24β–CH_3–Δ^{22}[e]	24β–CH_3–Δ^{22}[f]
Fragmentation						
M^+	-	-	-	-	396, 86	396, 99
M^+-CH_3	-	-	-	-	381, 3	381, 4
M^+–HOX (X = H or Ac)	366, 100	380, 100	378, 100	375, 100	378, 5	378, 7
M^+–HOX–CH_3	351, 13	365, 10	363, 5	363, 8	363, 100	363, 100
M^+–HOX–C_3H_5	325, 1	-	-	-	337, 45	337, 41
M^+–HOX–C_3H_7	-	337, 1	335, 2	335, 2	-	-
M^+–SC (SC = Side Chain)	-	-	-	-	271, 23	271, 30
M^+–HOX–SC	253, 20	253, 18	253, 29	253, 45	253, 59	253, 52
M^+–HOX–SC–C_3H_6	211, 13	211, 30	211, 11	211, 14	211, 36	211, 34
M^+–HOX–SC–C_3H_8	209, 2	209, 1	209, 5	209, 5	209, 4	209, 8

[a]7-Dehydrocholesteryl acetate synthesized from cholesterol.

[b]7-Dehydrocampesteryl acetate biosynthesized (*Tetrahymena pyriformis*) from 24α-methylcholesterol. We thank Dr. R.L. Conner and Mrs. J.R. Landrey for the incubations, a full report of which will be made elsewhere.

[c]24-Epiergosteryl acetate derived as described under footnote-b.

[d]Ergosteryl acetate from yeast.

[e]Ergosterol from yeast.

[f]Ergosterol from *Lycopodium complanatum*.

TABLE IV

Representative Proton Magnetic Resonance (PMR) Spectra of Tracheophyte Sterols in the Cholesterol Series[a]

Group at C–24 and side chain double bond	Chemical shift in ppm from TMS at 220 MHz								
	H[b]	α–CH_3[c]	β–CH_3[d]	α–C_2H_5[e]	β–C_2H_5[f]	α–CH_3–Δ^{22}[g]	β–CH_3–Δ^{22}[h]	α–C_2H_5–Δ^{22}[i]	β–C_2H_5–Δ^{22}[j]
Proton position									
C–18 (s)	0.68	0.68	0.68	0.68	0.68	0.69	0.70	0.70	0.70
C–19 (s)	1.01	1.01	1.01	1.01	1.01	1.02	1.01	1.01	1.01
C–21 (d) (J = 6)	0.91	0.91	0.92	0.92	0.93	1.00	1.01	1.02	1.03
C–26,27 (d) (J = 6)	0.87	0.77 0.85	0.77 0.86	0.81 0.84	0.81 0.83	0.82 0.83	0.82 0.83	0.80 0.85	0.79 0.85
C–28 (d) (J = 6)	-	0.80	0.78	-	-	0.91	0.91	-	-
C–29 (t) (J = 7-8)	-	-	-	0.85	0.86	-	-	0.80	0.81

[a]Abbreviations: s = singlet; d = doublet; t = triplet; J is given in Hz.

[b]New York fern.

[c]Main 24-methyl component of Christmas fern.

[d]Main 24-methyl-Δ^5-sterol from *Lycopodium complanatum.*

[e]*Kalmia latifolia.*

[f]*Chlorella ellipsoidea.*

[g]Diatoms, ref. 5.

[h]*Brassica rapa* seeds.

[i]Soybeans.

[j]*Chlorella ellipsoidea.*

TABLE V

Representative Proton Magnetic Resonance (PMR) Spectra of Tracheophyte Sterols and Standards in the Lathosterol Series[a]

Group at C−24 and side chain double bond	Chemical shift in ppm from TMS at 220 MHz								
	H[b]	α−CH_3[c]	β−CH_3[d]	α−C_2H_5[e]	β−C_2H_5[f]	α−CH_3−Δ^{22}[g]	β−CH_3−Δ^{22}[h]	α−C_2H_5−Δ^{22}[i]	β−C_2H_5−Δ^{22}[j]
Proton position									
C−18 (s)	0.53	-	0.54	0.54	0.54	-	0.54	0.55	0.55
C−19 (s)	0.80	-	0.80	0.80	0.80	-	0.80	0.80	0.80
C−21 (d) (J = 6)	0.92	-	0.92	0.93	0.93	-	1.01	1.02	1.03
C−26,27 (d) (J = 6)	0.87 0.86	- -	0.85 0.77	0.84 0.82	0.84 0.82	- -	0.83 0.82	0.85 0.80	0.85 0.79
C−28 (d) (J = 6)	-	-	0.78	-	-	-	0.91	-	-
C−29 (t) (J = 7)	-	-	-	0.85	0.86	-	-	0.80	0.82

[a]Abbreviations: s = singlet; t = triplet; J is given in Hz.

[b]From New York fern.

[c]Probably (MS) present in New York fern and *C. pepo* but insufficient amount for PMR.

[d]From *Chlorella emersonii* or signifies doublet.

[e]From *C. pepo* pericarp, or implies doublet.

[f]From *Chlorella emersonii.*

[g]Unknown.

[h]Synthetic; gift of H. Kircher.

[i]From *Spinacea oleracea.*

[j]From *Chlorella emersonii.*

TABLE VI

Proton Magnetic Resonance (PMR) Spectra of Sterols in the 7-Dehydrocholesterol Series[a]

Side chain	Chemical shift in ppm from TMS at 220 MHz				
	24–H[b]	24α–CH_3[c]	24α–CH_3–Δ^{22}[c]	24β–CH_3–Δ^{22}[d]	24β–CH_3–Δ^{22}[e]
C–18 (s)	0.61	0.62	0.63	0.63	0.63
C–19 (s)	0.94	0.95	0.95	0.95	0.95
C–21 (d) (J = 6)	0.94	0.93	1.03	1.04	1.04 (1.03 sh)
C–26,27 (d) (J = 6)	0.87	0.80 0.78	0.84 0.83	0.84 0.83	0.84 0.83
C–28 (d) (J = 6)	-	0.81	0.92	0.92	0.92 (0.91 sh)

[a]Abbreviations: s = singlet; t = triplet; J is given in Hz.

[b]Prepared synthetically from cholesterol.

[c]Prepared biosynthetically (*Tetrahymena pyriformis*) from 24α-methylcholesterol. We thank Dr. R.L. Conner and Mrs. J.R. Landrey for the incubations, a full report of which will appear elsewhere.

[d]Isolated from aerobically grown yeast or signifies doublet.

[e]Isolated from *Lycopodium complanatum*, or implies doublet in left-hand column.

moving component (15 mg) as described in a preliminary communication (13) was primarily ergosterol. This was demonstrated by its movement in GLC (RRT 1.30) and by its UV spectrum (λ_{max} 271, 282, and 293 nm) and mass and PMR spectra (Tables III and VI) when compared to that of ergosterol and 24-epiergosterol which was prepared biochemically from 24α-methylcholesterol (13). The PMR spectra of the epimers differ in the position of the signal for C-21 which is 2 Hz downfield in ergosterol. An upfield shoulder (intensity ca. 0.5) on the doublet for C-21 indicated the presence of 24-epiergosterol as a minor component. Only one other report (14) exists of ergosterol in a Tracheophyte and the configuration at C-24 was not determined. Not described in our preliminary paper was the next fraction (12 mg) which was a mixture of stigmasterol and 24-methylcholesterol in a ratio of 2:1, respectively. The ratio is based on the intensities of the signals for C-18 in the PMR spectrum. In the mass spectrum, the peaks for m/e 400 and 412 were equal, but stigmasterol, a $\Delta^{5,22}$-sterol, has a weaker peak for the molecular ion than do Δ^{5}-sterols due to enhanced allylic cleavage at the 17(20)-bond (Table II). The mass and PMR spectra were exact composites throughout of stigmasterol and 24-methylcholesterol. The 24α-configuration of the stigmasterol was demonstrated by comparison of the C-21 doublet with that of authentic samples of stigmasterol from soy beans and poriferasterol from *Chlorella ellipsoidea* (15) (Table IV). The doublet for C-21 and the triplet for C-29 were displaced downfield (1-2 Hz) in the latter (4,5). The triplet was interferred with by the 24-methylcholesterol in the *Lycopodium* sterol mixture, but the C-21 signal, being shifted due to the Δ^{22}-bond, was clearly visible as the downfield branch. It was exactly at the position found in stigmasterol. The configuration of the 24-methylcholesterol was also demonstrated by PMR spectroscopy. The diagnostic doublets (4) for C-21 and C-28 clearly showed the major epimer was 24β-methylcholesterol (Table IV). Indeed, there was only a suggestion (weak shoulders) of the presence of 24α-methylcholesterol. Also not reported in our preliminary paper (13) was the slowest moving component (19 mg) on Sephadex. It was very pure, identical with sitosterol in all respects (GLC, MS, and PMR), and different from its 24-epimer, clionasterol, in the PMR. Clionasterol was isolated from *Chlorella ellipsoidea*. Thus, the *Lycopodium* sterols were in descending order of their amounts, ergosterol, sitosterol, stigmasterol, and 22-dihydrobrassicasterol with small amounts of materials tentatively identified (PMR) as 24-epiergosterol and campesterol. The UV spectrum of the sterol mixture from the plant collected in the fall showed the typical absorption for the $\Delta^{5,7}$-system but less than from the summer sample.

The Ferns

In all four cases, the major sterol was sitosterol (GLC, MS, PMR) unaccompanied by stigmasterol (no m/e 412 peak occurring in the 24-methyl fraction with which it moves on Sephadex). The epimeric 24-methylcholesterols were always present as minor components (GLC, MS, and PMR for each fraction from each plant). The α/β-ratio for the latter deter-

mined by PMR in a manner described earlier (4) varied slightly, but campesterol was always present to a greater extent (ca. twice as much) than dihydrobrassicasterol. In the case of the Christmas fern, cholesterol (GLC, MS, and PMR) was also present. The New York fern similarly yielded cholesterol (GLC, MS, PMR). Thus, two of the ferns possessed the homologous Δ^5-series: 24–H, 24–CH_3, and 24–C_2H_5. The New York fern also contained by GLC the analogous Δ^7-homologs. Enriched fractions of the latter were obtained from the more polar half of the sterol band from alumina chromatography. They were obtained pure by sequential chromatography on thin layers of silica gel (removing Δ^5-sterols which moved faster) and a column of Sephadex (separating the homologs). Lathosterol moved the fastest. It was identified by GLC, MS, and PMR. 24α-Ethyllathosterol, moving the slowest, was similarly identified. The configuration at C-24 was demonstrated by comparison with authentic samples of 24α-ethyllathosterol from *Spinacea oleracea* (16) and 24β-ethyllathosterol from *Chlorella emersonii* (17). The PMR spectra of the epimeric standards (Table V) showed the expected (4,5) downfield shifts in the singals for C-21 and C-29 yielding clearly different absorption patterns which, incidentally, spectroscopically confirms that *S. oleracea* has sterols with the opposite configuration from that in *C. emersonii*. The spectrum of the fern sterol was identical with that of the 24α-epimer from *S. oleracea*. While there was a suggestion of 24-methyllathosterol in the GLC of the appropriate chromatographic fractions, there was too little to identify.

The ferns, in summary, contained 24α-ethylcholesterol, no stigmasterol, lesser amounts of 24α-methylcholesterol which was in greater quantity than 24β-methylcholesterol, and, in certain cases, still smaller amounts of cholesterol. Δ^7-Sterols appearing only in the New York fern were in lesser amount than Δ^5-sterols, but, as in the latter case, the 24α-ethyl component was in greater amount than the component with a 24-H-atom. No seasonal variant was observed (GLC) in the relative constituents with the New York fern which was examined both in early summer and the fall. From a summer sample, 58 mg of sterol from 100 g of wet wt of tissue was obtained. The sterols were in the following ratio: 24–C_2–Δ^5 to 24–C_1–Δ^5 to 24–H–Δ^5 to 24–C_2–Δ^7 to 24–H–Δ^7: 1.0 to 0.18 to 0.11 to 0.08 to 0.07. The Christmas fern yielded 29 mg of sterol per 100 g wet wt of tissue. The 24–C_2–Δ^5–, 24–C_1–Δ^5–, and 24–H–Δ^5– components were present in a ratio of 1.0 to 0.18 to 0.10. An unidentified component (RRT 1.81) which probably was 24α-ethyllathosterol was also present at about the level of cholesterol.

Ginkgo biloba

The sterol of the maidenhair tree has been reported (18) to be 24-ethylcholesterol (beleived but unproven to be the 24α-epimer, sitosterol, identified by m.p., GLC, and IR) containing a trace of its 22-dehydroderivative (thought to be stigmasterol identified only by GLC). We separated the two components by chromatography on lipophilic Sephadex for further study. The major component, moving the slower, had the same rate of movement in GLC as sitosterol. It possessed a PMR spectrum identical with that of sitosterol (4,5) and distinctly different from that of the epimeric clionasterol (4,5) proving the suspected 24α-ethyl configuration to be correct. The component moving faster on Sephadex had a retention time in GLC the same as 24-methylcholesterol which is similar to that of stigmasterol. Its mass spectrum, however, was identical to that of 24-methylcholesterol rather than the suspected stigmasterol. Based on the molecular ions at m/e 398, 400, and 412, the sample was 83% 24-methylcholesterol, 10% "stigmasterol", and 5% "brassicasterol." Minor components also were evident from enhanced fragmentations at m/e 314, 299, 172, and 229. It is not obvious what they were due to, although they are found with 25(27)-dehydroclionasterol. The PMR spectrum was identical with that of an authentic 60:40 mixture (4) of 24α- and 24β-methylcholesterol and different from either alone (4,5). A weak "doublet" (J = 7) from a minor component appeared at 0.98 ppm which, however, does not agree with expectation for stigmasterol, brassicasterol, or 25(27)-dehydroclionasterol. Cholesterol was also apparent in the mixed sterols by GLC. The major sterols of the maidenhair tree are, thus, in descending order of amount, 24α-ethyl-, 24α-methyl, and 24β-methylcholesterol and probably cholesterol itself with trace amounts of unidentified materials of molecular weights 412, 398, and perhaps others. The leaves yielded 55 mg of sterol per 100 g wet wt. The 24–C_2–Δ^5–, 24–C_2–$\Delta^{5,22}$–, 24–C_1–Δ^5–, and 24–H–Δ^5–sterols were present in a ratio of 1.0 to trace to 0.10 to 0.10.

Kalanchoe daigremontiana

The 4-desmethylsterol mixture was composed of two sterols (RRT of 1.42 and 1.54). They were separated on Sephadex. The faster moving (RRT 1.42) exhibited the mass spectrum [m/e 410 (M^+, 19%), 395 (M^+–CH_3, 5%),

392 (M^+-H_2O, 4%), 381 ($M^+-C_2H_5$, 14%), 377 ($M^+-CH_3-H_2O$, 4%), 363 ($M^+-C_2H_5-H_2O$, 11%), 325 (M^+–85, 7%), 314 ($M^+-C_7H_{12}$, 9%), 309 (M^+–101, 11%), 300 (M^+–110, 41%), 271 (M^+–side chain–2H, 100%), 255 (M^+–side chain–H_2O, 58%), 253 (M^+-H_2O–side chain–2H), 239 (8%), 215 (17%), and 213 (M^+-H_2O-side chain–C_3H_6, 26%]; PMR spectrum [0.70 (s, C-18), 0,83 (t, J = 7.5 Hz, C–29], 1.03 (d, J = 6.5 Hz, C–21), 1.01 (s, C-19), and 1.65 ("d", J = 1 Hz, C–26) ppm; m.p. (151 C); and IR spectra (ν_{max} 965 and 890 cm^{-1}) expected of 25(27)-dehydroporiferasterol [previously isolated from *Clerodendrum campbelli* from which its structure and configuration were demonstrated (11)]. The slower moving component (RRT 1.55, m.p. 135-136 C, ν_{max} 890 cm^{-1}) in mass spectroscopy showed m/e 412 (M^+, 100%), 397 (M^+-CH_3, 25%), 394 (M^+-H_2O, 17%), 381 ($M^+-C_2H_7$, 5%), 379 ($M^+-CH_3-H_2O$, 28%), 328 (M^+–84, 18%), 314 ($M^+-C_7H_{14}$, 45%), 299 ($M^+-C_8H_{17}$, 50%), 281 (15%), 271 (M^+–side chain–2H, 75%), 255 (M^+–side chain–H_2O, 33%), 253 (M^+–side chain–H_2O–2H, 20%), 231 (M^+–side chain–C_3H_6, 38%), 229 (M^+-side chain–C_3H_8, 33%), and 213 (M^+-H_2O–side chain–C_3H_6, 67%). In the PMR spectrum, signals appeared at 0.68 (s, C–18), 0,80 (t, J = 7.5 Hz, C–29), 0.91 (d, J = 6 Hz, C–21), 1.01 (s, C–19), and 1.57 ("d", J = 1 Hz, C–26) ppm. These data prove the compound to be either the 22-dihydro derivative [25(27)-dehydroclionasterol] of the faster moving component or the analogous derivative [25(27)-dehydrositosterol] of the latter's 24-epimer. The 24β-configuration of both fast and slow moving components was demonstrated as follows. The mixed 4-desmethylsterols from alumina chromatography of the neutral lipid were converted to their corresponding 3,5-cyclo-6β-yl methyl ethers by treatment with fused KOAc in methanol at reflux for 2 hr. The chromatographically (Al_2O_3) purified but unseparated pair of methyl ethers (RRT 0.46 and 0.50) was hydrogenated (PtO_2, dioxane/HOAc) to give a single 3,5-cyclosteryl methyl ether (RRT 0.50). Retrorearrangement (fused ZnOAc, HOAc, 6 hr at reflux) gave a single Δ^5-sterol which had a m.p. (136-137 C) and PMR spectrum identical with clionasteryl acetate (from *Chlorella ellipsoidea*). Clionasteryl and sitosteryl acetates have distinctly different melting points and PMR spectra (4,5). Our *Kalanchoe* sterols must, therefore, both have had the 24β-ethyl configuration and been 25(27)-dehydroclionasterol [clerosterol previously isolated from *Clerodendrum infortunatum* (10) but without proof for the configuration at C–24] and 25(27)-dehydroporiferasterol (*trans*-22-dehydroclerosterol). Other $\Delta^{25(27)}$-sterols, viz. cyclolaudenol and the 24β-methyl analog (codisterol) of 25(27)-dehydroclionasterol, occur in algae (19,20), and the former in Tracheophytes, e.g., ferns (21). The 25(27)-designation rather than 25(26) is made by the following analogy. When 25(27)-dehydroporiferasterol was labelled from 2-^{14}C-MVA, the methylene carbon was found to be unlabelled (11). If we designate C–26 as the labelled carbon derived from 2-^{14}C-MVA, then cyclolaudenol, codisterol, clerosterol, and the latter's 22-dehydro derivative become $\Delta^{25(27)}$-sterols on the assumption that the same biosynthetic stereospecificity occurs in both algal and Tracheophyte systems and is the same for the first as well as second C_1-transfer. To our knowledge, this work represents the first proof of configuration at C–24 for clerosterol, the first time both clerosterol and its 22-dehydro derivative have been found in the same plant, and only the second time either has been reported. From the very watery *K. daigremontiana* plants was isolated 5.4 mg of sterol per 100 g of wet wt. Each of the two components comprised about one-half of the total amount.

It is also interesting that we observed a 1 Hz split in the "singlet" for C–26 in both $\Delta^{25(27)}$-sterols. Tentatively, we interpret this to result from a coupling with the H-atom on C–24 which implies C–26 lies between the H-atom and C–28 (as shown in Table I) in the preferred conformation. In turn, since a higher multiplet was not observed, the two H-atoms on C–28 must lie on the side opposite to C–26 with C–29 projecting away ("up") from the remainder of the side chain.

Kalmia latifolia

The only sterol found was sitosterol (m.p., GLC, MS, PMR) at a level of 59 mg/100 g wet wt. Since a ketone in even larger amount (78 mg/100 g) was present (moving on alumina just after the hydrocarbons), we also examined it to determine whether it was steroidal. While it was not, it is an interesting enough compound to report on.

The ketone possessed an RRT of 2.81, a.m.p. greater than 230 C, and a carbonyl peak (ν_{max} 1790 cm^{-1}) in the IR indicative of the pentacyclic triterpenoid friedelin already known (21-23) to be present in the genus *Rhododendron* of the family Ericaceae to which *Kalmia* belongs. The spectral and other properties described in what follows agree with this assignment of structure. The mass spectrum of the ketone indicated a molecular weight (M^+

97%) of 426 for $C_{30}H_{50}O$. In addition to loss of CH_3 (m/e 411, 31%), fragmentations occurred at and near the ring junctions yielding C_5H_9O (m/e 341, 17%), $M^+-C_9H_{16}$ (302, 62%), $M^+-C_{10}H_{19}$ (287, 24%), $M^+-C_{11}H_{21}$ (273, 98%), $M^+-C_{13}H_{24}$ (246, 62%), $M^+-C_{14}H_{26}$ (232, 60%), $M^+-C_{15}H_{28}$ (218, 81%), and a combination of $M^+-C_{16}H_{29}$ and $C_{14}H_{21}O$ (205, 100%) resulting from clevage through the center of ring C. Upon reduction with $LiAlH_4$, the carbonyl group disappeared (IR), and the product fragmented (MS) in nearly the same way as the ketone except that the fragments occurred two mass units higher, the cleavage in ring A was insignificant, and a peak at m/e 205 remained with one at 207. The reduction product also showed M^+-H_2O and $M^+-H_2O-CH_3$. In the PMR spectrum of the ketone, two multiplets near 2.26 and 2.36 ppm for the H-atoms on C–2 and C–4, five singlets for three protons at 0.71, 0.85, 0.94, 1.04, and 1.16 ppm, and one singlet for six protons at 0.99 ppm were observed. A doublet also appeared at 0.87 ppm (J = 6.5 Hz). These signals account for theoretical expectation for the eight methyl groups in friedelin. The doublet is clearly from the methyl group on C–4. Partial assignment of the remaining signals can be made from the spectrum of the corresponding alcohol. While the peaks at 0.85, 0.94, 0.99, and 1.16 remained unchanged, the doublet moved to 0.93 (J - 6.5 Hz) in the alcohol and the other two singlets (0.71 and 1.04 ppm) moved to 0.96 and 1.00 ppm. They are, therefore, probably the closest ones to C–3 and represent the CH_3 groups at C–5 and C–9, respectively. The two peaks at 0.99 ppm are probably the *gem*-dimethyl group and the one at 1.16 ppm (which does not appear in the spectra of lanosterol or cycloartenol) is probably the CH_3-group at the *cis*-juncture between rings D and E. The spectral properties confirm the structural assignment as friedelin. To our knowledge, these spectra have not previously been described for this unique cyclization product of squalene oxide. While friedelin occurs in a variety of botanical families (24), we have not observed it in any of the other families examined in this or our earlier work (4).

Cucurbita pepo

In view of the presence of 24α- and 24β-alkylsterols and the unusual absence of Δ^5-sterols in pumpkin seeds (6-9), we made an exceptionally careful study of the mature plant. The pericarp (after removal of seeds and associated structures) and the leaves of plants collected in early autumn were examined separately. In addition to separations on Sephadex, the 4-desmethylsterol fraction (Al_2O_3-chromatography) from the pericarp was acetylated and further separated by chromatography on a thick layer of silica gel impregnated with 10% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6, v/v). The sterol fraction from the leaves was chromatographed directly as the free alcohol on silica gel impregnated with 10% $AgNO_3$ in the solvent system chloroform-ligroin-acetone (75:23:4, v/v). A monoene fraction (Fraction 1, with the same rate of movement as sitosterol) and two diene fractions [with the rates of movement, respectively, of stigmasterol (Fraction 2) and fucosterol (Fraction 3)] were eluted for further study. The analytical data are recorded as follows. R_f (SiO_2) refers to the rate of movement of the alcohol in thin layer chromatography (TLC) on silica gel in chloroform. R_s (RP) refers to the rate of movement of the alcohol relative to cholesterol in reversed phase TLC (25). R_f ($AgNO_3$) is the rate of movement of the acetate after one development (unless otherwise noted) on a thin layer of silica gel impregnated with 10% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6, v/v). All compounds showed only "end absorption" in the ultraviolet spectrum. The value quoted is at 220 nm in ethanol. The Liebermann-Burchard test (LB) was performed in acetic anhydride-sulfuric acid (19:1, v/v) at room temperature. A color reaching maximum intensity in a minute or less is described as "fast," and one requiring 30 min as "slow." The following data were obtained with authentic steroids. Sitosterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.79; R_f ($AgNO_3$) 0.23; RRT 1.54; LB slow, blue, and its Δ^7-isomer (24α-ethyllathosterol) showed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet. Spinasterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.94; R_f ($AgNO_3$) 0.19; RRT 1.53; LB fast, violet. Fucosterol exhibited R_f (SiO_2) 0.18; R_s (RB) 1.01; R_f ($AgNO_3$) 0.14; RRT 1.63, LB slow, blue. Isofucosterol possessed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.10; RRT 1.68, LB slow, blue. Stigmasterol showed R_f (SiO_2) 0.18; R_s (RP) 0.29; RRT 1.34.

Fraction 1 (24α-ethyllathosterol) from the pericarp possessed R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet; ϵ 1,500; m.p. 142-144 C (152-155 C as acetate); ν_{max} 800, 830 cm^{-1}; m/e for the acetate 456 (M^+), 441 (M^+-CH_3), 396 (M^+-HOAc), 381 (M^+-CH_3-HOAc), 255 (M^+-HOAc–side chain), 213 (M^+–60–side chain–42).

Fraction 2 (24α-ethyl-22-dehydrolathosterol, spinasterol) from the pericarp possessed

R_f (SiO_2) 0.18; R_f ($AgNO_3$) 0.18; RRT 1.54; LB fast, violet; ϵ 1,400; m.p. 159-161 C (172-177 C as acetate); ν_{max} 800, 830, 970 cm^{-1}; m/e for the acetate 454 (M^+), 439 (M^+-CH_3), 411 ($M^+-C_3H_7$), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 351 ($M^+-C_3H_7$–HOAc), 315 (M^+–side chain), 273 (M^+–side chain–42).

Fraction 3 from the pericarp was not investigated except by gas liquid chromatography (GLC) which showed two major (93%) components (ratio of 1:1), RRT 1.79 and 1.95 agreeing with the values for peposterol and Δ^7-avenasterol as discussed below.

Fractions 1 and 2 from the leaves were not well separated perhaps due to different relative amounts compared to the pericarp fractions. They were combined and submitted to preparative GLC. One fast and one slow moving component were observed equivalent, respectively, to fractions 2 and 1. The latter 24ξ-ethylsterol possessed R_f (SiO_2) 0.18; R_s (RP) 0.81; R_f ($AgNO_3$) 0.23; RRT 1.80; LB fast, violet; ϵ 3,000; m.p. 141-143 C; m/e for the alcohol 414 (M^+), 399 (M^+-CH_3), 396 (M^+-H_2O), 381 ($M^+-CH_3-H_2O$), 273 (M^+–side chain), 255 (M^+-H_2O–side chain) 213 (M^+-H_2O–side chain) 213 (M^+-H_2O–side chain–42); δ 0.53 (s, C–18), 0.79 (s, C–19), 0.92 (d, C–21), 0.85 (poorly resolved multiplet, C–26,27,29) ppm at 100 MHz. The fast moving component in GLC (24ξ-ethyl-22-dehydrolathosterol, spinasterol) possessed R_f (SiO_2) 0.18; R_s (RP) 0.94; R_f ($AgNO_3$) 0.18; RRT 1.55; LB fast, violet; ϵ 1,900; m.p. 158-161 C; ν_{max} 800, 830, 970 cm^{-1}, m/e for the alcohol 412 (M^+), 397 (M^+-CH_3), 369 ($M^+-C_3H_7$), 351 ($M^+-C_3H_7-H_2O$), 273, 272 and 271 (M^+–side chain–0, 1, and 2 H–atoms), 255 (M^+–side chain–H_2O), 213 (M^+–side chain–H_2O–42).

Fraction 3 from the leaves showed a peak with a shoulder on the leading side in GLC. The two components were separated as the acetates on preparative chromatoplates of silica gel containing 15% of $AgNO_3$ in the solvent system hexane-chloroform-acetic acid (75:25:0.6) by two or more developments. The one moving slower on the chromatoplate (*trans*-24-ethylidenelathosterol, Δ^7-avenasterol) possessed R_f (SiO_2) 0.18, R_f (15% $AgNO_3$ 2 passes of solvent) 0.11; RRT 1.98 (2.11 as acetate); LB fast, violet; m.p. 139-141 C (acetate); ν_{max} 800, 830 cm^{-1}; m/e; for the acetate 454 (M^+), 439 (M^+-CH_3), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 356 ($M^+-C_7H_{14}$), 315 (M^+–side chain), 314 (M^+–side chain–1), 313 (M^+–side chain–2), 273 (M^+–side chain–42), 255 (M^+–side chain–HOAc), 253 (M^+–side chain–HOAc–2); δ for the acetate 0.53 (s, C–18), 0.80 (s, C–19), 0.97 doublet (C–21, C–26, C–27), 1.59 (d, J = 6 Hz, C–29), 2.01 (CH_3 of acetyl), 5.15 (m, H on C–7 and C–28) ppm at 100 MHz. The faster moving component (which we shall call peposterol) showed R_f (SiO_2) 0.18; R_f (15% $AgNO_3$, 2 passes) 0.15; RRT 1.75 (1.89 as acetate); LB fast, violet; m.p. 135-137 C (acetate); ν_{max} 800, 930 cm^{-1}; m/e for acetate 454 (M^+), 439 (M^+-CH_3), 394 (M^+–HOAc), 379 (M^+-CH_3–HOAc), 356 ($M^+-C_7H_{14}$), 315 (M^+–side chain), 314 (M^+–side chain–1) 313 (M^+–side chain-HOAc–2), 273 (M^+–side chain–42), 255 (M^+–side chain–HAOc), 253 (M^+–side chain–HOAc–2); δ 0.51 (s, C–18), 0.80 (s, C–19), 0.90 (d, J = 6 Hz, C–21), 0.93 (t, J = 6-7 Hz, C–29), 1.56 (sh 1.59, C–26 and C–27), 2.01 (CH_3 on acetyl) ppm at 100 MHz.

The α-configuration at C–24 was demonstrated by PMR at 220 MHz in a later set of experiments in which the pericarp 4-desmethylsterols derived from the free and esterified pools were chromatographically separated on Sephadex. The two main components had m.p.'s (142-143 C and 157-159 C), RRTs (1.79 and 1.53) and mass and PMR spectra which were identical with 24α-ethyllathosterol and its *trans*-22-dehydro derivative, spinasterol, respectively, isolated from spinach, and the PMR spectra differed from the 24β-epimers isolated from *Chlorella emersonii*. The PMR spectrum of the leaf 24ξ-ethyllathosterol isolated by argentation chromatography was taken earlier at 100 MHz. Unfortunately, the resolution, while confirming the Δ^7-assignment (C–18 signal at 0.53 ppm), was insufficient for configurational determination and too little sample was left for examination at 220 MHz. Presumably the configuration is the same as in the pericarp specimens. Similarly the PMR spectrum of peposterol (one of the minor components from leaf "Fraction 3") was taken not only at 100 MHz but on a very small sample. The resolution of the multiplets left something to be desired. Only six methyl peaks (other than for acetyl at 2.01 ppm) were seen. The ones at 0.51 and 0.80 ppm were clearly singlets for C–18 and C–19, in the Δ^7-series. The other four experimental peaks (0.86, 0.93, and 1.00 and 1.56 ppm) agree with the interpretation of singlets and multiplets given in the paragraph describing "Fraction 3" indicating the sterol is probably 24-ethyl-24(25)-dehydrolathosterol. However, until a larger sample is available for further study we prefer to make the structural assignment only tentatively. A sterol assigned this structure has already been obtained (26) from sunflower but was mixed with Δ^7-avena-

sterol. The interesting signal from C–29 in the PMR spectrum is not recorded, and the doublet from C–29 in the Δ^7-avenasterol confuses the interpretation of the peaks in the 1.6 ppm region where C–26 and C–27 should give signals. However, it is claimed (26) that two singlets appeared at 1.57 and 1.66 ppm for the $\Delta^{25(27)}$-sterol. In our case, only the former was seen as a strong peak (1.56 ppm), although a weak multiplet did appear from 1.70 to 1.74 ppm. Thus, unfortunately in neither the present nor previous (26) case is a completely adequate structural analysis possible.

In addition, the following compounds were present based on weak extra peaks for M^+ in the mass spectra of the samples of 24α-ethyllathosterol isolated by argentation chromatography: lathosterol, m/e 386 ca. 0.5 of m/e 414 (leaf); 24ξ-methyllathosterol, m/e 400 2.0% of m/e 414 (leaf) and 5.0% of m/e 414 (pericarp). The pericarp 24ξ-methyllathosterol (presumably of the α-configuration) was further identified by the presence of an 8% component showing a peak at RRT 1.45 contaminating the 24α-ethyllathosterol (RRT 1.80). Had the minor component been the Δ^5-isomer, the RRT would have been 1.29. From the pericarp fractions appearing in the alumina column prior to the appearance of 4-desmethylsterols, two triterpenes were isolated, one had the same mass spectrum as authentic cycloartenol (as the acetate, m/e 468, 453, 408, 393, 365, 357, 339, 297, 286, 271, and 175). The other (as the acetate, m/e 468, 453, 408, 393, 301, 289, 241, 229, 218, 205, 191, and 189) was not further studied. The ester and free alcohol fractions of the pericarp chromatographically separated prior to saponification were also investigated separately in a similar manner. The same 4-desmethylsterols were obtained. Moreover, no Δ^5-sterols could be detected either in the ester or the free sterol pools by MS, GLC, PMR, or Liebermann-Burchard Test.

C. pepo thus contains 24α-ethyl-*trans*-22-dehydrolathosterol and 24α-ethyl-lathosterol in descending order of amount with much smaller quantities of lathosterol, 24ξ-methyllathosterol (presumably in the 24α-series), *trans*-24-ethylidenelathosterol, and (tentatively) 24-ethyl-24(25)-dehydrolathosterol. The two principal components were present at levels (mg/100 g of wet tissue) of 8.0 and 7.0, respectively, in the leaf and of 1.8 and 1.7 in the much more watery pericarp. In *S. oleracea* leaf, the values were 4.3 and 3.5, respectively. *trans*-24-Ethylidenelathosterol amounted to about 3 in *C. pepo* leaves which also contained about 1 of the "$\Delta^{24(25)}$"-isomer, 0.1 of the 24-methyl component, and 0.04 of lathosterol.

DISCUSSION

In our previous (4) and present work, we have examined thirteen families in the Phylum Tracheophyta. Using derived hydrocarbons, Mulheirn (27), has also examined *Zea mays* bringing to fourteen the number of families studied with PMR at 220 MHz. The plants range from the subphylum Lycopsida characterized by simple vascular tissue, poorly developed roots, and sporangia through the Filicopsida with a more complex vascular system, but simple roots and no flower, fruit, or seed to the true seed bearing plants (Pteropsida) culminating in the class Angiospermae with flowers, fruit, and the most complex of plant vascular tissue extending through large and well developed roots, stems, and leaves. While the number of families examined represents only a few of those existing, the evolutionary range gives validity to some taxonomic and phylogenetic considerations. They are particularly interesting with sterols as a tool, because all algae and all Tracheophytes, so far as is known, biosynthesize sterols, and because there are cogent reasons (28) for believing sterols perform a vital function as architectural components in the lipid leaflet of membranes.

It is clear from our work that there are two extreme types of plant in terms of the configuration at C–24. In Category I are plants containing exclusively or primarily 24α-alkylsterols, while in Category II are those containing exclusively or primarily 24β-alkylsterols (Table VII). Furthermore, this categorization depends on ontogeny. Seeds of the family Cucurbitaceae contain 24β-ethylsterols (6-8) together with, as recently demonstrated with *C. pepo* (9), 24α-ethylsterols in smaller amount (ca 23%). However, we have shown that in mature tissue of this plant, only 24α-ethylsterols are present in consequential amount. This appears to be an evolutionary recapitulation, since sterols of the great majority of the investigated nonvascular plants (algae and fungi) contain only 24β-alkylsterols. Our work also shows that an intermediate type exists in *Lycopodium complanatum* in which the 24-ethylsterol possesses the α-configuration exclusively as do plants of Category I, but, in the 24-methylsterols, the β-configuration is dominant as in plants of Category II. This chemical intermediacy correlates with primitive vascularization in the Lycopsida and suggests that increasing proportions of 24β-alkylsterols may exist in the liverworts and mosses which are "higher" than algae in being Embryophytes but "lower" than Tracheophytes in not being vascularized. Configurational data are not yet

TABLE VII

Classification of Tracheophyte Families by Side Chain[a]

Category I (Primarily 24α-Alkyl)[b]	Intermediate[c]	Category II (Primarily 24β-Alkyl)[b]
Pteropsida		
Angiospermae		
Leguminosae		
Pisum sativum (s);		
Glycine max (s)		
Ericaceae		
Kalmia latifolia (ℓ)		
Brassicaceae		
Brassica oleracea (ℓ)		
Chenopodiaceae		
Spinacea oleracea (ℓ)		
Cucurbitaceae		Cucurbitaceae
Cucurbita pepo (p,ℓ)		*Cucurbita pepo* (s) and others
Magnoliaceae		Verbenaceae
Liriodendron tulipifera (ℓ)		*Clerodendrum infortunatum* *Clerodendrum campbellii*
Podophyllaceae		Crassulaceae
Podophyllum peltatum (ℓ)		*Kalanchoe daigremontiana* (ℓ)
Gymnospermae		
Pinaceae		
Pinus pinea (s, en, e)		
Ginkgoaceae		
Ginkgo biloba (ℓ)		
Filicopsida		
Polypodiaceae		
Polystichum acrostichoides (f)		
Dennstaedtia pucti lobula (f)		
Dryopteris (Thelypteris) *noveboracensis* (f)		
Osmundaceae		
Osmunda cinnamomea (f)		
	Lycopsida *Lycopodium complanatum* (ℓ)	

[a]Abbreviations used: e = embryo of seed; en = endosperm of seed; f = fronds; ℓ = leaves; p = pericarp of fruit; s = whole seeds.

[b]The plants listed are those examined in our present and previous (4) work. Included are also the only other two families known (10,11) to contain 24β-ethylsterols. In all of the plants in Category I the tissues examined have only 24-ethylsterols in the 24α-series, and the 24-methylsterols, when present, were always a minor fraction and principally, but not exclusively, of the α-configuration. In the one plant (*K. daigremontiana*) with the 24β-ethylsterols which we examined, except for a very small amount (ca. 2%) of unidentified sterol, all of the sterol had the 24β-ethyl structure. In *Cucurbita pepo* seeds 77% of the sterol has the 24β-ethyl structure (6). Reports (10,11) on the two species of Clerodendrum do not make clear the percentage of epimers, but we presume the absence of mention of 24α-sterols implies the 24β-ethylsterols to be dominant.

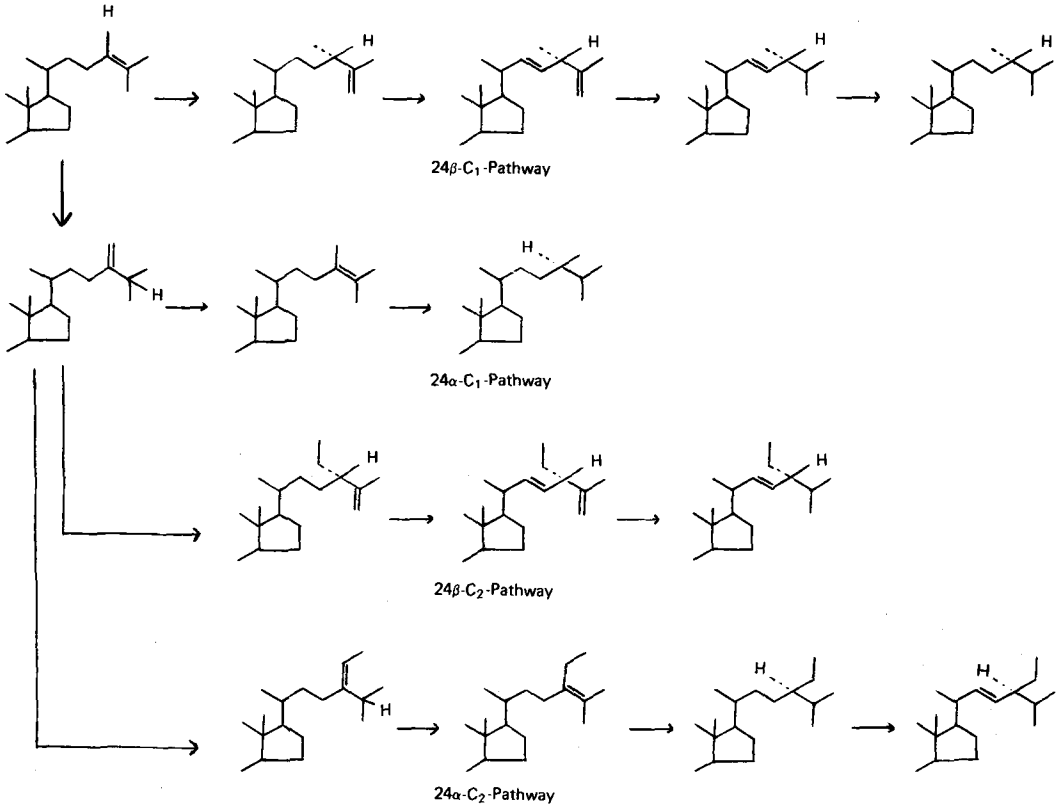
[c]In *L. complanatum* the only 24-ethylsterol possessed the α-configuration but most of the 24β-methylsterol possessed the 24β-configuration. The ratio of 24α-alkylsterol to 24β-alkylsterol was ca. 1.6 to 1.0. Thus the plant was closer to Category I than to II.

available on these interesting organisms, but at least, in the case of the mosses, 24ξ-methyl- and 24ξ-ethylcholesterol are reported to be present (29-31).

In addition to variations in the side chain, the type and degree of unsaturation in the nucleus function as taxonomic and phylogenetic markers. Again, there are two extreme types of Tracheophyte: Category A containing primarily Δ^5-sterols and Category B with Δ^7-sterols. The Cucurbitaceae are the most well studied of Category B. The seeds of several genera in this family have already been found (6-9) to possess only Δ^7-sterols, and we have now found that the fruit and leaves of *C. pepo* also contain only Δ^7-sterols. *C. pepo* has, therefore, been examined at all stages of ontogeny and is unquestionably in Category B throughout its development. *S. oleracea* leaves are similarly known to contain Δ^7-sterols (16). We have confirmed this and find further that there are no detectable Δ^5-sterols. *S. oleracea* is, therefore, another plant in Category B at least in the mature stage. Based on the sterols, in

Scheme I

Probable Pathways to Tracheophyte Sterols



leaves or in the oils of seeds of e.g., *Camellia japonica* and *Thea sinensis* (32,33), these plants similarly belong to Category B as probably do *Acacia* species (34). Most other plants studied, such as the others examined by us in the present and previous work (4), are of Category A. It is interesting that *Ginkgo* nuts were recently examined (40) and found, as were the male leaves in our work, to contain only Δ^5-sterols in the homologous series (in increasing amount: 24–H, 24–CH_3, and 24–C_2H_5 with some 24-ethyl-Δ^{22}-sterol).

From the foregoing discussion, it is apparent statistically that most plants are of Categories I and A, i.e., containing principally 24α-alkyl-Δ^5-sterols. For this reason, we believe the 24α-alkyl-Δ^5-structure, and especially the one with the 24α-ethyl group commonly represented by sitosterol, constitutes the most highly evolved of the alternative structures. This is given further credence by the apparent evolutionary recapitulation in *C. pepo* from Category II to I, by the intermediate status of *L. complanatum*, and by the sterols of algae (Category II).

The biosynthetic pathway in plants of Category II is thought to proceed through the introduction of a $\Delta^{25(27)}$-bond at the time of C_1-transfer to C–24 or C–28 (11). Our finding that *Kalanchoe daigremontiana* contains 24β-ethyl-$\Delta^{25(27)}$-sterols is further evidence for such a pathway (Scheme I), and it is presumably more primitive than the one to 24α-alkyl-sterols. The latter probably occurs through $\Delta^{24(25)}$-sterols (35-37). Our tentative detection of 24-ethyl-24(25)-dehydrolathosterol and its association in mature *C. pepo* plants with the $\Delta^{24(28)}$-isomer (Δ^7-avenasterol) and with 24α-ethyllathosterol and its 22-dehydro derivative (spinasterol) is consistent with a route (Scheme I) in which the second C_1-transfer yields a $\Delta^{24(28)}$-sterol which is isomerized to the $\Delta^{24(25)}$-isomer followed by reduction to the 24α-ethyl end-product. The existence of the $\Delta^{24(25)}$- and $\Delta^{25(27)}$-routes to the epimeric sterols are given further substantiation by the finding (M.L. McKean and W.R. Nes, unpublished observations) that 24-tritiolanosterol leads in *Pinus pinea* to labelled 24-methylcholesterol (presumably the 24β-component) but to unlabelled 24α-ethylcholesterol, while

2-^{14}C-MVA produces more label in the latter than in the former.

If we extend our categorization to include plants with dominant sterols bearing the $\Delta^{5,7}$-diene structure (Category C), it becomes possible to include the algae under the system. Thus, *Chlorella ellipsoidea* (15) is Category II-A, *Chlorella emersonii* (17) is II-B, and *Chlorella simplex* (38) is II-C. It is interesting to note that the blue-green *Phormidium luridium*, which is much more primitive then the *Chlorella* species each of which is of a single category, is of a mixed category (A,B, and C) (39). Similarly, *Lycopodium complanatum* is mixed (A and C), and, relative to the Pteropsida and most of the Filicopsida examined where single double bond categories exist, *L. complanatum* is more primitive. The full significance of these mixed types remains to be demonstrated, but they would appear to represent plants in which the sterol pathway is not kinetically evolved to the point found in others of a single double bond category. By analogy to animals, the pathway Δ^7 to $\Delta^{5,7}$ to Δ^5 presumably operates in plants. In most plants, as in animals, this pathway is kinetically completed with little or no steady-state concentration of intermediates, i.e., the plants are of single category (A,B, or C). In view of the data presented here and elsewhere, we belive it plausible to believe, if tentatively, that plants in Category B, *C. pepo* for instance, represent an evolutionary line which did not develop the gene (or its expression) for the Δ^5-dehydrogenase, that plants in Category C are in a line lacking the Δ^7-reductase, and that plants of a mixed category (or ones bordering on it, e.g., the New York fern with some Δ^7-sterols in a larger pool of Δ^5-sterols) reflect the quite different condition of having the enzymes, and therefore the genetics, but not having the fully developed regulatory genes for perfect kinetic control. An alternative explanation for Category B amd C plants is retroevolution in which the genetics for the appropriate enzyme has been lost. Unfortunately, no obvious way exists to discriminate between these possibilities at the present time, but on the assumption that retroevolution has not taken place it is possible to assess the phylogenetics of the plants examined. This means that in the order of evolution of the unsaturation in ring B, we expect Categories C, B, and A, while for the configuration we expect II and I. A plant of Category II-C would therefore be the most primitive with which, for instance, *Chlorella simplex* agrees in its morphology relative to, say, *Pisum sativum*, an angiosperm of Category I-A, and we would place *C. simplex*, *C. emersonii*, and *C. ellipsoidea* in the relative (direct or parallel) evolutionary order in which they are mentioned.

Two quite different taxonomic and phylogenetic relationships have been suggested in the literature for the angiosperms by Hutchinson (12) and Cronquist (41). Our chemotaxonomy based on the sterols offers a way of examining them. From morphologic, geographic, and other parameters, both Hutchinson and Cronquist place Magnoliales (including *Liriodendron tulipifera*) as the most primitive order of flowering plant closely associated with Ranales of the Hutchinson system which Cronquist [at least in so far as the species (*Podophyllum peltatum*) we examined is concerned] regards as Ranunculales. Hutchinson considers Magnoliales to have given rise to woody dicots and Ranales to herbaceous dicots and perhaps also to monocots, while in the Cronquist system the woody and herbaceous divisions are not recognized. Since the family *Cucurbitaceae* and the examined species of Theales are of Category B and both Magnoliales and Ranales (Ranunculales) are of Category A, the sterol data correlate with neither system in the sense of a direct progenitor role of Magnoliales for these Category B plants. It seems to us that the sterols may indicate a parallel evolution with rather than a direct evolution from Magnoliales. Similarly, Hutchinson's suggestion of the evolution of Saxifragales from Ranales is not verified, since *Kalanchoe daigremontiana* (Saxifragales) is of Category II and *Podophyllum peltatum* (Ranales) is of Category I, nor is his suggestion of the line to Ericales through Theales from Magnoliales verified, since *Kalmia latifolia* (Ericales) is of Category I-A while representatives of Theales, e.g., the genus *Camellia*, are of Category I–B (32,33). The Theales to Ericales (and Capparales including *Brassica*) line of Cronquist similarly is not consonant with our data. On the other hand, there are aspects of the Cronquist system with which our data either do agree or with a slight change in his system would agree. He places *Cucurbita* in Violales emanating from Theales and both are, in fact, of Category I-B in the mature stage. He also places *Crassulaceae* (which includes *Kalanchoe*) in the Rosales with Leguminosae (which includes *Pisum* and *Glycine*), and the former is supposed to have given rise to the latter with which the change from Category II (*Kalanchoe*) to Category I (*Pisum* and *Glycine*) would agree. *Clerodendrum* (Category II-A) in Lamiales is placed above Rosales. Again the sterol data agree, but only if the evolutionary line to *Clerodendrum*

bifurcates (at or before *Crassulaceae* of Category II-A) before the formation of Leguminosae of Category I-A. Unfortunately, despite the coincidence of the data in and above Rosales with the previous classifications, the sterol data do not support the origin of Rosales itself (in the Cronquist system) from Magnoliales (Category I-A) in view of his placing Crassulaceae (Category II-A) in the order. In Hutchinson's system, *Crassulaceae* is supposed to have emanated from Ranales which agrees no better.

The detailed disagreements delineated between sterol structure and previously suggested phylogenetics should not, however, obscure more general agreements. Thus, the line of Cronquist from Magnoliales to Ranunculales to *Spinacea* in Carophyllales is verified (all Category I-A). Furthermore, the sterol structures are consistent with, while not proving, that many angiosperms are higher and could have arisen from the gymnosperms, since the two species of the latter studied were of Category I-A as are most of the angiosperms studied. Similarly, the Filicopsida are of Category I-A. This tells us that evolution of the pathway to 24α-ethylsterols in the Δ^5-series was accomplished quite early chronologically and is not associated with the presence of characters such as flowering. In summary, if retroevolution did not occur in the sterol pathway which at the present is not amenable to experimental verification, some of the angiosperms e.g., Cucurbitaceae, could not have arisen from a line comprised by Filicopsida, Gymnospermae, and Magnoliales and must have diverged very early, but the sterol data do not conflict with the thesis that many flowering plants could have arisen from Magnoliales.

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Resolution of Molecular Species of Intact Serine and Ethanolamine Phosphatides by Argentation Chromatography of Their Trifluoroacetamides[1]

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ABSTRACT

An effective resolution of intact phosphatidylserines on the basis of unsaturation has been achieved by conventional argentation thin layer chromatography (TLC) following trifluoroacetylaction. The trifluoroacetamides are prepared by treatment with trifluoroacetic anhydride or N-methyl-bis-trifluoroacetamide. The acetamides are resolved with chloroform-methanol-water (65:25:4, v/v/v) on Silica Gel G containing 20% silver nitrate. Subfractions with 0-6 double bonds per molecule were obtained for the phosphatidylserines of pig and ox brain, pig erythrocytes, rat liver, and rabbit skeletal muscle. The preparation of trifluoroacetamides is also advantageous for the silver ion fractionation of phosphatidylethanolamines. The method is applicable to metabolic studies of molecular species using radioactive precursors of neutral lipids, phosphorus, and nitrogenous bases.

INTRODUCTION

In recent years chromatographic systems have been developed for the resolution of intact phosphatidylcholines (1), phosphatidylethanolamines (2), and phosphatidylinositols (3) into different classes of unsaturation, which has led to the recognition of a metabolic heterogeneity of these phosphatides. None of these methods, however, has proved adequate for the separation of the phosphatidylserines, which in addition to the phosphate hydroxyl and the amino function, also possess a polar carboxyl group. Consequently, studies of the metabolism of the molecular species of the serine phosphatides have been impossible.

Like phosphatidylethanolamine, phosphatidylserine forms a dinitrobenzamide which possesses excellent solvent partition properties especially following further methylation (4), but this derivative is difficult to recover from the reaction mixture when present in small amounts. Much more readily prepared and recovered are the trifluoroacetamides of phosphatidylserine in which the polar amino group is masked resulting in greatly improved chromatographic properties, as already demonstrated for amino alcohols (5) and amino acids (6,7). As a background for studies of the metabolism of molecular species, the present report describes the preparation of trifluoroacetamides from synthetic and natural phosphatidylserines and phosphatidylethanolamines along with their separation into different classes of unsaturation by $AgNO_3$-TLC.

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MATERIALS AND METHODS

Synthetic 1,2-dipalmitoyl *sn*-glycerol-3-phosphorylserine along with the phosphatidylserine of pig and ox brain were purchased from Serdary Research Laboratories, London, Canada. The trifluoroacetic anhydride was obtained from Eastman Kodak Co., Rochester, NY and the N-methyl-bis-trifluoroacetamide from Pierce Chemical Co., Rockford, IL. The methylene chloride and other solvents and reagents were of Fisher Certified Reagent grade. [$^{1-14}$C] Acetic anhydride (spec. act. 10 mCi/mg) was from New England Nuclear, Boston, MA.

Isolation of Phosphatidylserine

Total lipid extracts of egg yolk, pig erythrocytes, rat liver, and rabbit skeletal muscle were prepared as described (8). A combined phosphatidylserine-phosphatidylinositol fraction was isolated by thin layer chromatography (TLC) on Silica Gel H using chloroform-methanol-acetic acid-water (25:15:4:2, v/v/v/v) as the developing solvent (9). The phosphatides were eluted (2) from the gel with chloroform-methanol-acetic acid-water (50:39:1:10, v/v/v/v), the combined extracts washed with 1/3 volume of 4 M NH_4OH, and the solvents evaporated to small volume. Pure phosphatidylserine was isolated (10) by TLC of the mixed serine and inositol phosphatides in the solvent system chloroform-acetone-methanol-acetic acid-water (5:2:1:1:0.5, v/v/v/v/v). The phosphatidylserine moved ahead of the phosphatidylinositol and was recognized by the pink

color that it gave with a ninhydrin spray (11). Phosphatidylserine containing L-serine-U-^{14}C was isolated from rat liver 5 hr after intravenous injection of L-serine-U-^{14}C. The above chromatographic system was also employed to purify the commercial samples of phosphatidylserine.

Isolation of Phosphatidylethanolamine

Phosphatidylethanolamine of egg yolk, rat liver, and rabbit skeletal muscle was prepared by TLC using chloroform-methanol-water (65:25:4, v/v/v) as the developing solvent (8). This phosphatide was eluted from the silica gel with chloroform-methanol (2:1, v/v) and washed with 1/5 volume of water.

Preparation of Trifluoroacetamides

The glycerophospholipids (2-5 mg) were dissolved in 1 ml of ice-cold methylene chloride and were treated with an equal volume of a 10% solution of trifluoroacetic anhydride in ice-cold methylene chloride. The reaction was allowed to proceed at ice temperature for 5 min. At this time, 4 ml of ice-cold chloroform-methanol (1:1, v/v) were slowly added followed by 1.2 ml of ice-cold 4 N ammonium hydroxide. The chloroform phase was separated, passed through a small column of anhydrous sodium sulfate, and concentrated under nitrogen. Alternately the trifluoroacetamides were prepared by addition of an excess (0.3 ml/10 mg lipid) of N-methyl-bis-trifluoroacetamide to the methylene chloride solution of the phosphatide at room temperature (22 C) and allowing the reaction to proceed overnight (16 hr). The reagent was removed at 37 C under nitrogen. The reaction products were isolated by TLC using chloroform-methanol-water-conc. ammonia (65:25:3:1, v/v/v/v) as the developing solvent. The various lipid fractions were located by spraying the plates with 2,7-dichlorofluorescein and viewing them under ultraviolet light. The trifluoroacetamides were specifically located on the basis of a positive reaction to mercury-molybdic acid (11) and a negative reaction to ninhydrin (11) sprays. The trifluoroacetamides were extracted from the gel as described above for the native phosphatides.

Preparation of Radioactive Acetamides

Radioactive acetamides were prepared from synthetic phosphatidylserine under the general conditions employed for obtaining the trifluoroacetamides. The phosphatides (1-2 mg) were reacted with an excess of [1-^{14}C] acetic anhydride (spec. act. 10 mCi/mg) at room temperature, the products isolated as above, and the amount of radioactivity incorporated into the reaction products per mole of phosphatide was determined by scintillation counting and gas liquid chromatography (GLC) of fatty acids on aliquots of the material (12).

Subfractionation of Trifluoroacetamides

The trifluoroacetamides of the serine and ethanolamine phosphatides were resolved on the basis of degree of unsaturation by $AgNO_3$-TLC. Silica gel plates (20 x 20 cm, 0.5 mm thick layer) containing 20% $AgNO_3$ were prepared using a stainless steel applicator (Desaga Brinkmann) and after air drying were activated at 110 C for 1 hr. The trifluoroacetamides were applied as narrow bands from a chloroform solution about 2.5 cm from the lower edge of the plate and the plates were developed with chloroform-methanol-water (65:25:4, v/v/v) as the solvent system. The location of the separated bands was detected by spraying the plates with dichlorofluorescein and viewing them under ultraviolet light. The various bands were identified and quantitated by analyzing the fatty acid composition following transmethylation in the presence of silica gel (13) using heptadecanoic acid as internal standard. Alternately, the lipids were first extracted from the gel by washing with chloroform-methanol-acetic acid-water as described for the isolation of the total acetamides, and the lipid fractions transmethylated in the conventional manner (8).

Hydrolyses with Phospholipase A_2

Positional analyses of the faty acids in the phosphatidylserine and phosphatidylethanolamine were carried out as described (14).

Mass Spectrometry

The mass spectra of the glycerophospholipids and their trifluoroacetylation products were obtained under the conditions described for the analysis of bile acid conjugates (15). The mass spectrometer was a Varian Mat CH-5 single focusing instrument coupled to a Varian 620 computer and direct probe inlet, which could be temperature programmed from ambient to 300 C. The mass spectrometer was operated at an ionization voltage of 70 eV, an accelerating voltage of 3000 V, electron emission energy of 100 uA, and an ion source temperature of 270 C. The bulk of the solute to be analyzed evaporated from the probe at about 200 C. Scanning was made at 4 s/decade and at a resolution of 800-1000.

Infrared Spectrometry

Infrared spectra of phospholipids were ob-

tained on a Perkin-Elmer 237 B Infrared Spectrophotometer. The wave numbers were calibrated using a polystyrene film. Lipids were dissolved in methylene chloride (10-50 mg/ml) and were read in 0.2 mm path-length cavity cells.

Nuclear Magnetic Resonance (NMR) Spectroscopy

The NMR spectra were obtained on a Varian Model T-60 spectrometer. The lipids were dissolved in $CDC1_3$ (10-50 mg/ml) and the spectra determined relative to tetramethylsilane at 10 ppm (16) at ambient temperature (33 C).

Polarimetry

Optical rotations were measured in a Perkin-Elmer Model 141 Polarimeter using 1 ml x 1 dm cells. The lipids were dissolved in chloroform (0.6-2.0%) and were read before and after desalting. For desalting, 0.1 to 0.2 ml of glacial acetic acid was added to 5-25 mg of the phospholipid (17). After standing at room temperature for 15 min, chloroform (5 ml) was slowly added, and the lipids washed with water, adding sufficient methanol to break any emulsion.

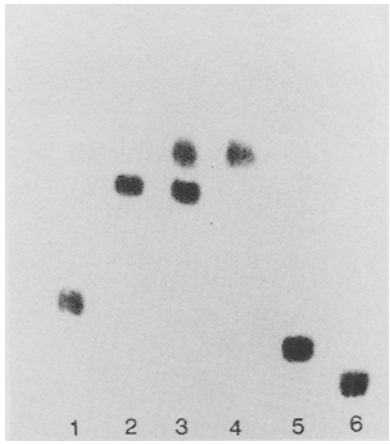


FIG. 1. Thin layer chromatography (TLC) separation of ethanolamine and serine phosphatides and their trifluoroacetylation products. 1, phosphatidylethanolamine; 2, trifluoroacetamide of phosphatidylethanolamine; 3, a mixture of the trifluoroacetamide of phosphatidylethanolamine (lower spot) and the azlactone derivative of the trifluoroacetamide of phosphatidylserine (upper spot); 4, azlactone derivative of trifluoroacetamide of phosphatidylserine; 5, trifluoroacetamide of phosphatidylserine; 6, phosphatidylserine. TLC conditions: adsorbent, Silica Gel H; solvent, chloroform-methanol-water-conc. ammonium hydroxide (65:25:3:1 v/v/v/v). Visualization of spots by staining with phosphomolybdic acid.

RESULTS AND DISCUSSION

Preparation and Characterization of Trifluoroacetamides

The trifluoroacetylation of phosphatidylethanolamine served as a model reaction for the derivatization of phosphatidylserine. The reaction of phosphatidylethanolamine with trifluoroacetic anhydride was complete within a few minutes and gave a single product, which was negative to a ninhydrin and positive to a phosphate spray (Fig. 1). The trifluoroacetamide (Rf 0.71) migrated ahead of the unreacted phosphatide (Rf 0.57) when chromatographed in chloroform-methanol-water-conc. ammonia (65:25:3:1, v/v/v/v). The trifluoroacetamide of phosphatidylethanolamine failed to show absorption characteristic of NH_2 group at 1625 cm^{-1}, which was present in the infrared spectrum of the parent compound (18). Since tha amide bond at 1736 cm^{-1} of the trifluoroacetamides is not resolved from the glycerol ester C=O stretching at 1733 cm^{-1} (18,19), the trifluoroacetamide of phosphatidylethanolamine had more intense absorption in this frequency region than the parent compound. The NMR spectra of the phosphatidylethanolamine and its trifluoroacetamide were similar. The trifluoroacetamide of phosphatidylethanolamine had $(\alpha)_D^{25}$+6.0 in chloroform (c, 1.0) which after desalting changed to $(\alpha)_D^{25}$0.0 in chloroform (c, 0.6) compared to $(\alpha)_D^{25}$+4.1 in chloroform (c, 1.2) for the parent compound in the salt-free form. According to Baer and Buchnea (20), the salt-free dioleoylphosphatidylethanolamine has $(\alpha)_D^{25}$+6.0 in chloroform (c, 7.0).

Under similar conditions, trifluoroacetylation with the anhydride yielded two derivatives of phosphatidylserine (Fig. 1). Both the major (Rf 0.33) and the minor (Rf 0.80 products migrated ahead of the unreacted phosphatidylserine (Rf 0.21) when run in the above TLC system. Reaction with N-methyl-bis-trifluoroacetamide gave only one derivative corresponding to the major product (Rf 0.33) in the anhydride reaction. All the products gave negative tests with ninhydrin and a positive test for phosphate. On the basis of the time course of appearance of the two products in the anhydride reaction (Fig. 2), the more polar major product appeared to be the precursor of the minor product. The sole or the major product of trifluoroacetylation of the phosphatidylserine was therefore assumed to be the trifluoroacetamide. The infrared spectrum of the

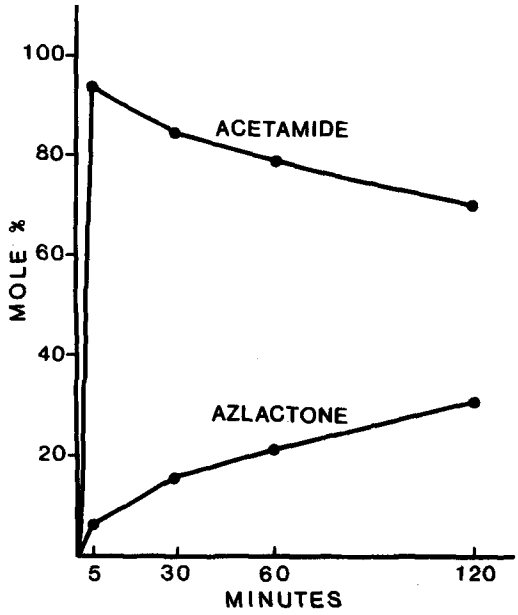


FIG. 2. Time-course of formation of the trifluoroacetamide and azlactone of [U-^{14}C]-serine labeled phosphatidylserine. Each point represents a single estimate of the amount of the trifluoroacetyl derivative formed in same reaction mixture. Reaction monitored by change in radioactivity.

trifluoroacetamide of phosphatidylserine was consistent with the absence of a free amino group and the presence of an amide group as discussed for the trifluoroacetamide of phosphatidylethanolamine. Little difference was noted in the infrared spectra of the major and minor products of phosphatidylserine trifluoroacetylation. The NMR spectrum of the trifluoroacetamide of phosphatidylserine was similar to that of the parent compound. The minor product of trifluoroacetylation of phosphatidylserine exhibited an absorption peak in the NMR spectrum at 5.9 ppm, which was absent from the NMR spectra of the parent compound and its trifluoroacetamide. The trifluoroacetamide of phosphatidylserine had $(\alpha)_D^{25}$+21 in chloroform (c, 1.4) which remained unchanged on desalting. The parent phosphatidylserine had $(\alpha)_D^{25}$+11 in chloroform (c, 1.7), which on desalting changed to $(\alpha)_D^{25}$-11 in chloroform (c, 1.0) and compared favorably to $(\alpha)_D^{25}$-13.6 in chloroform (c, 3.0) recorded by Baer and Maurukas (17) for the salt-free distearoylphosphatidylserine. The minor product had $(\alpha)_D^{25}$-12 in chloroform (c, 1.0), which on desalting changed to $(\alpha)_D^{25}$ 0.0 in chloroform (c, 0.5). Direct probe mass spectrometry gave fragmentation patterns which were consistent with a simple trifluoroacetamide structure for the major or sole product of trifluoroacetylation of phosphatidylserine. Figure 3 shows the direct probe mass spectrum of the trifluoroacetamide of the tetraenoic phosphatidylserines, which constitute the major species in rat liver. The spectrum gives a base peak for the RCO+74 fragment (21) and readily detectable M-98 ion (20% of base peak for tetraenes and 3-4% of base peak for the monoenes), which represented a loss of the trifluoroacetyl groups plus an additional hydrogen. Another large fragment represented the loss of an acyl group from M-98. The latter fragments still retained the serine residue. The M-98 ions were not seen in the mass spectra of the original phosphatidylserine, its minor transformation product, or of phosphatidylethanolamine and its trifluoroacetamide. The highest mass ion seen in all of

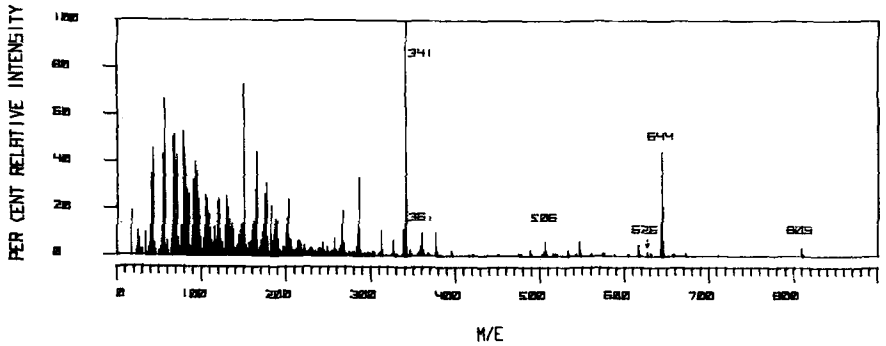


FIG. 3. Direct probe mass spectrum of the trifluoroacetamide of the tetraenoic phosphatidylserines of rat liver. The m/e 809 fragment corresponds to the $(M-98)^+$ ion of the stearoyl-arachidonoyl species whereas the ion at m/e 506 results from a further loss of the arachidonoyl acyloxy radical. The fragment at m/e 644 is an ion having the molecular formula of stearoyl-arachidonoyl glycerol which upon loss of one molecule of water gives a fragment at m/e 626. Fragments at m/e 341 and 361 correspond to the $(RCO+74)^+$ ions of stearate and arachidonate, respectively. Some corresponding fragments are seen for the palmitoyl-arachidonoyl species also present in the trifluoroacetamide form.

TABLE I

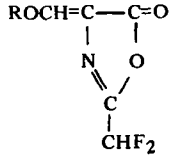
Fatty Acid Composition[a] of Selected Phosphatidylethanolamines and Phosphatidylserines and of Corresponding Trifluoroacetamides

Fatty acids	Phosphatidylethanolamine				Phosphatidylserine		
	Egg yolk		Rat liver		Rat liver		
	Original	Acetamide	Original	Acetamide	Original	Acetamide	Azlactones
	moles%						
14:0	0.5	0.8					
16:0	22.1	22.5	23.4	22.7	4.1	4.7	3.9
16:1	0.8	0.7					
18:0	26.2	24.5	24.2	26.3	47.0	43.5	44.6
18:1	17.9	16.7	5.0	4.0	2.6	3.9	3.7
18:2	12.3	12.7	5.6	4.5	1.9	1.9	1.9
20:2	1.5	1.3					
20:3	0.8	1.6	0.7	0.7	0.7	0.6	0.9
20:4	11.4	11.5	20.9	20.6	24.7	24.1	23.4
20:5			2.2	2.3	2.0	1.3	2.3
22:3	0.9	0.9					
22:4	2.5	3.2					
22:5			1.8	1.8	1.0	3.4	1.9
22:6	3.1	3.5	16.3	17.1	15.9	16.6	17.4

[a]Representative compositions from two to four separate derivatizations and determinations of fatty acids. Values of less than 0.5% have been rejected.

the latter was the dehydrated diacylglycerol ion.

On the basis of co-chromatography (Fig. 1), the by-product appearing in the reaction of the serine phosphatide with trifluoroacetic anhydride could not be due to decarboxylation of the serine residue. Furthermore, since acetylation with radioactive acetic anhydride indicated only one mole of acetate per mole of the major and minor reaction products, the faster moving component could not be attributed to a higher acetyl derivative. A likely possibility, however, was the internal anhydride or azlactone formation. Carter (22) has reviewed the formation of azlactones from acylamides of amino acids and their dehydration products. Furthermore, Weygand and Glockler (23) have reported that in the presence of an excess of trifluoroacetic anhydride the α-amino acids yield N-trifluoroacetylamino acid anhydrides or azlactones. Finally, Sheehan and Duggins (24) have prepared the azlactones from a substituted α-amino acid trihaloacetamide and have suggested appropriate structures. On the basis of the above studies, the azlactone of phosphatidylserine trifluoroacetamide may be represented as follows:



where R is the phosphatidyl moiety.

This structure is consistent with the chromatographic properties of the secondary reaction product of phosphatidylserine with trifluoroacetic anhydride. As seen from Figure 2, the formation of the secondary reaction product can be minimized by limiting the time of reaction with the anhydride. It can be avoided completely by preparing the trifluoroacetamide by means of the N-methyl-bis-trifluoroacetamide.

Table I shows good agreement between the fatty acid composition of the trifluoroacetamides and that of the corresponding original phosphatide. The total fatty acid composition of the phosphatidylserine of rat liver is similar to that reported by Wood and Harlow (25), while those of the phosphatidylethanolamines of the egg yolk and rat liver are similar to those previously described by Holub and Kuksis (26) and Arvidson (2), respectively.

$AgNO_3$-TLC of Trifluoroacetamides of Phosphatidylethanolamine

Figure 4 shows the separation obtained for rat liver phosphatidylethanolamine before and after trifluoroacetylation. While the underivatized phosphatide gives a reasonable separation of the major bonds, the trifluoroacetamides show a resolution into seven distinct subfractions very much like that realized for the diacylglycerol acetates analyzed previously (27), to which these fractions also correspond in fatty acid composition. A comparable resolution of the molecular species was obtained for the trifluoroacetamides of egg yolk phospha-

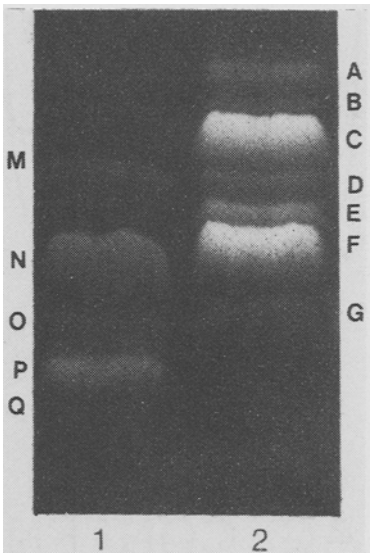


FIG. 4. $AgNO_3$-TLC separation of rat liver phosphatidylethanolamine. 1, original phosphatidylethanolamine: (M) monoenes, dienes and trienes; (N) tetraenes; (O) pentaenes; (P) hexaenes, and (Q) heptaenes; 2, trifluoroacetamides: (A) monoenes and dienes; (B) trienes; (C) tetraenes; (D) pentaenes I; (E) pentaenes II; (F) hexaenes; (G) heptaenes. $AgNO_3$-TLC conditions: adsorbent, Silica Gel G impregnated with 20% silver nitrate; solvent, chloroform-methanol-water (65:25:4, v/v/v).

tidylethanolamine, which also yielded fatty acid compositions corresponding in all respects to those obtained for the corresponding diacylglycerol acetates examined previously (26). Trifluoroacetylation of the ethanolamine phosphatides was also compatible with the analysis of the plasmalogens. Figure 5 shows the fatty acid and dimethylacetal patterns for the various unsaturation classes of the phosphatidylethanolamines of rabbit skeletal muscle. Complete recoveries were obtained for the palmityl and stearyl aldehydes in the appropriate TLC bands. This resolution provided the basis of identification of the molecular species of the plasmalogens of rabbit skeletal muscle reported elsewhere by Marai and Kuksis (28).

Sundler and Akesson (29) have reported on the $AgNO_3$-TLC separation of the molecular species of phosphatidylethanolamine as the N-acetyl-O-methyl derivatives, which yield resolutions comparable to those reported for the trifluoroacetamides. Methylation with diazomethane, however, is experimentally dangerous and may be accompanied by transmethylation (30) and diazomethanolysis (31). The trifluoroacetamides are sufficiently nonpolar not to require methylation for satisfactory chromatography.

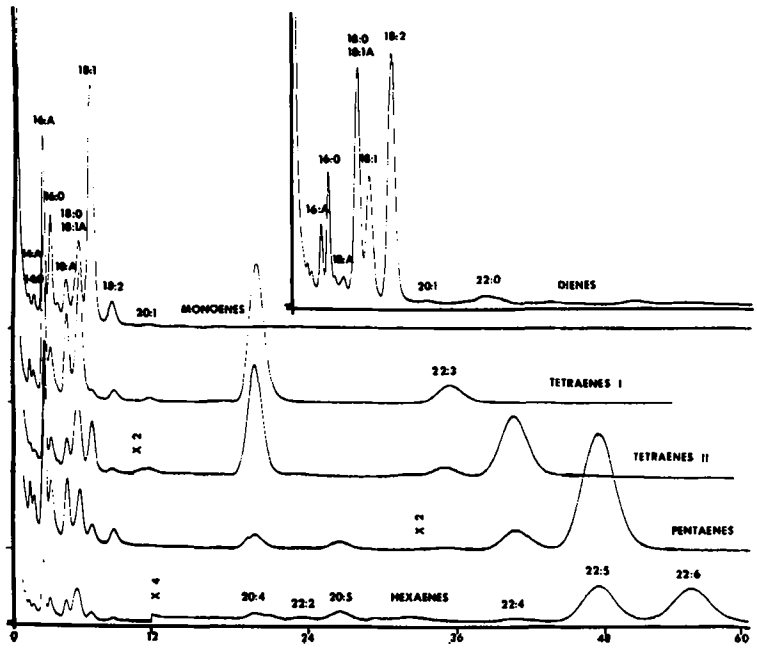


FIG. 5. Fatty acid and dimethylacetal composition of various $AgNO_3$-TLC bands of trifluoroacetamides of phosphatidylethanolamine of rabbit skeletal muscle. $AgNO_3$-TLC conditions as in Figure 4. Gas liquid chromtography conditions as given in text.

$AgNO_3$-TLC of Trifluoroacetamides of Phosphatidylserine

Figure 6 shows the separation of the two trifluoroacetylation products of phosphatidylserine by argentation TLC. A total of six bands may be recognized for each derivative. The azlactones, which were less polar, gave the more complete resolution. Table II gives the proportions of the subfractions along with the quantitative composition of the component fatty acids. The reconstituted composition of the fatty acids is very similar to that of the original phosphatide indicating an essentially complete recovery of all the fractions. The estimated molar proportions of the acids approximate the ratios required for pairing the saturated and unsaturated fatty acids within each subfraction. The high proportions of the tetraenes and hexaenes, and the predominance of stearate within these two chemical classes indicate unequivocally that the rat liver phosphatidylserines consist mainly of stearoylarachidonoyl (18:0, 20:4) and stearoyl docosahexaenoyl (18:0, 22:6) species.

Composition of Molecular Species of Phosphatidylserine

Table III lists the molecular species calcu-

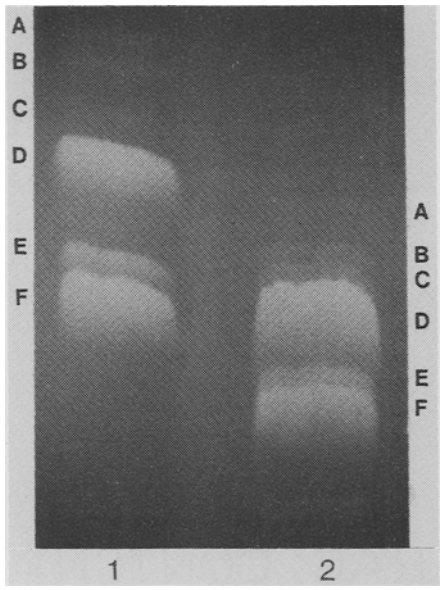


FIG. 6. $AgNO_3$-TLC separation of rat liver phosphatidylserine. 1, azlactones; 2, trifluoroacetamides; (A) monoenes; (B) dienes; (C) trienes; (D) tetraenes; (E) pentaenes; (F) hexaenes. $AgNO_3$-TLC conditions as given in Figure 4.

TABLE II

Fatty Acid Composition of Various Chemical Classes of Rat Liver Phosphatidylserine

Fatty acids	Total		Chemical classes[a]					
	Original	Reconst.	Monoenes	Dienes	Trienes	Tetraenes	Pentaenes	Hexaenes
				moles %				
Trifluoroacetamides								
16:0	4.5	4.8	32.7	4.7	3.4	3.6	6.6	5.1
18:0	49.5	51.3	29.8	44.2	51.8	52.7	54.0	50.3
18:1	3.9	4.0	37.8		36.6	1.5	7.2	1.6
18:2	1.6	1.2		51.1				
20:3	0.4	0.4			8.2			
20:4	22.1	19.5				42.2	9.1	0.4
20:5	2.5	2.2					19.3	1.5
22:5	1.3	0.3					3.7	
22:6	14.1	16.1						41.0
Azlactones								
16:0	5.4	5.4	16.9	6.3	10.0	4.7	4.9	5.0
18:0	47.0	50.1	31.5	40.7	48.0	49.4	53.5	53.0
18:1	2.8	3.4	45.6	21.9	7.9	1.7	1.0	
18:2	1.5	1.7	6.0	31.1	9.5		1.5	
20:3	0.4	0.5			24.5			
20:4	23.7	19.6				44.2	2.6	
20:5	2.4	2.0					22.5	
22:5	1.2	1.2					13.9	
22:6	15.5	16.0						42.0

[a]The chemical classes of the trifluoroacetamide and azlactone fractions were made up as follows: (moles %) monoenes 2.4, dienes 3.2, trienes 3.2, tetraenes 43.9, pentaenes 8.7, and hexaenes 38.6, respectively. Representative compositions from three separate derivatizations, separations, and determinations of fatty acids.

TABLE III

Major Molecular Species[a] of Selected Phosphatidylserines

Molecular species	Rat liver[b]	Pig erythrocytes[c]	Rabbit muscle[d]	Pig brain[d]	Ox brain[d]
		moles %			
Monoenes					
16:0 18:1	1	1	6	4	
18:0 18:1	2	32	23	93	92
18:0 20:1				3	5
Dienes					
18:1 18:1		33			
16:0 18:2	1		4		
18:0 18:2	3	10	24		
Trienes					
18:1 18:2		13			
18:0 20:3			2		
18:0 22:3			12		
Tetraenes					
16:0 20:4	4				
18:0 20:4	39	1	8		
18:0 22:4			2		
Pentaenes					
18:1 20:4	1	1			
18:0 20:5	4				
18:0 22:5	2		8		
Hexaenes					
16:0 22:6	3				
18:0 22:6	35				

[a]Molecular species making up less than 1% each have been rejected.
[b]Values calculated from data given in Table II.
[c]Average of two separate analyses.
[d]Single determinations.

lated from the above experimental results for the serine phosphatides of rat liver, and from similar results for rabbit skeletal muscle, ox and pig brain, and pig erythrocytes. The major species of the rat liver phosphatidylserines are the tetraenes (18:0, 20:4; 39%) and the hexaenes (18:0, 22:6; 35%) while the other species make up a total of 25%. The value for 18:0, 22:6 species is about twice as high (35%) as the value (11-18%) reported by Wood and Harlow (25) on the basis of GLC analyses of the total diacylglycerol moieties of rat liver phosphatidylserine. The phosphatidylserines of the rabbit skeletal muscle contain nearly equal proporations of monoenes (18:0, 18:1; 23%) and dienes (18:0, 18:2; 24%), with lesser amounts of trienes (18:0, 22:3; 12%) and polyenes (18:0, 20:4; 8%; 18:0, 22:5; 8%). Freshly prepared phosphatidylserines of pig erythrocytes contained monoenes (18:0, 18:1; 32%) and dienes (18:0, 18:2; 10%; 18:1, 18:1; 33%) in nearly equal amounts, while a commercial preparation of phosphatidylserines from pig brain contained mainly monoenes (18:0, 18:1; 93%). In all instances, the phosphatidylserines were characterized by a high proportion of stearic acid among the saturated fatty acids, with palmitic acid making up only a small proportion of the total fatty acids. The proportions of linoleic acid were also extremely low in all the phosphatidylserines examined. There are isolated instances, however, where high proportions of linoleic acid have been reported (32) in phosphatidylserine, but it is not known to what extent this is due to dietary influences and to an experimental contamination.

It should be noted that N-acyl phosphatidylethanolamine has been reported in plant seeds (33,34) and N-acyl glycerophosphorylethanolamine in the lipids of hog stomach (35), while the N-acyl phosphatidylserine has been tenta-

tively identified in sheep red blood cells (36). Such components would be expected to have chromatographic properties similar to the acetyl derivatives prepared in this study. However, the natural N-acyl derivatives would have been removed from the common phosphatides during their initial isolation.

The above study completes the silver nitrate subfrationations of the major mammalian glycerophospholipids. Further investigations with labeled phosphate, serine, glycerol, and fatty acids using the intact phosphatidylserines are now feasible as are determinations of the metabolic relationships among the molecular species of phosphatidylserine and other glycerophospholipids (S.K.F. Yeung and A. Kuksis, in preparation).

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Specific Distribution of Fatty Acids in the Triglycerides of Rainbow Trout Adipose Tissue. Influence of Temperature[1]

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ABSTRACT

In the trout, the unsaturated fatty acids are preferentially located in the β-position and the saturated fatty acids in the α-position of triglycerides. This fatty acid distribution is retained even with diets containing lard. The fish are, therefore, able to modify completely the fatty acid distribution of dietary triglycerides. There is no retention of the β-monoglyceride structure during the biosynthetic processes. However, the modification of the dietary fatty acid distribution by the trout seems to be more difficult at 18C than at 10C.

INTRODUCTION

The hypothesis according to which the structure of β-monoglycerides is retained through the food chain was formulated largely on the basis of the specific distribution pattern of fatty acids on the hydroxyl groups of glycerol observed in marine animals (1). This hypothetical rule, although reflecting some experimental facts, obviously did not give any biological or biochemical reason for the existence of a particular triglyceride structure in the living organisms which start the food chain. According to this rule, the preferential acylation of the β-position of glycerol by the polyunsaturated fatty acids in the cod, lobster, and scallop could be explained by the existence of an analogous distribution pattern of fatty acids on glycerol in plants and marine plankton. In 1964, this hypothesis was supported by studies in the lobster, cod and trout, and in the rat (2). The animals were fed labeled palmitic acid, as α-dioleyl-β-palmityl glycerol [α-monoacyl-glycerol refers to 1 (or 3)-monoacyl-L-glycerol and β-monoacyl-glycerol to 2-monoacyl-L-glycerol. The α-β nomenclature will be used for triglycerides.]. The authors noticed that this acid was retained in the β-position of glycerol in the tissues of marine animals only.

As a result of a stereospecific analysis of the triglyceride fatty acids, Brockerhoff (3,4) attempted to classify animal species according to glyceride structure (5,6). He found that in a great number of species the chain length and desaturation of the fatty acids seemed to govern the distribution between β-position of glycerol, on the one hand, and α-position, on the other hand. Thus, in mammals, except pig and some marine mammals, the shortest and most unsaturated chains showed a tendency to occupy the β-position. However, in fish and invertebrates, the specific distribution pattern of the fatty acids on glycerol was explained by the rule according to which the β-monoglyceride structure of the dietary triglycerides is retained.

In 1967, Brockerhoff and Hoyle (7) took up the problem again. They fed the animals double-labeled triolein, one label in the α-position and one in the β-position of glycerol. In this experiment, the hypothesis seemed to be valid for the lobster, but, in the trout, the percentage of ^{3}H in the β-position decreased from 80% in the food to 50% in the tissues and the percentage of ^{14}C increased from 5% to 40% tending to contradict the hypothesis.

Our purpose was to investigate to what extent trout kept at different temperatures are able to change the position of the β-acyl chains of the ingested triglycerides. To make easier the interpretation of the results, the fatty acid composition of the tissue studied had to be as simple as possible. In a preliminary experiment, we showed that the fatty acid composition of the perigastric adipose tissue (P.A.T.) was rapidly and strongly affected by composition of the dietary fat making possible the desired modification. The perigastric tissue is homogeneous and it is easily found on the greater curvature of the trout's stomach. However, we have recently observed (unpublished data) a slight superficial contamination with diffuse pancreatic tissue near the stomach curvature. This compact fat cannot be confused with the perivisceral adipose tissue. Its phospholipid content is negligible compared with that of the triglycerides and, therefore, does not affect the interpretation of the results. Because of all these considerations, this tissue was chosen for

[1]Presented in part at the 13th World Congress of the International Society for Fat Research, Marseille, France, 1976.

TABLE I

Composition of the Diets[a]

Component	Complete diet	Divided into diets	
		Without linolenate	With linolenate
Casein	50[b]	37.5	12.5
Normal or interesterified Lard	10	7.5	2.5
Maize starch	35	26.25	8.75
Minerals	2	1.5	0.5
Vitamins – F.A.O.	2	1.5	0.5
Linolenic acid	1	0	1
	100	74.25	25.75

[a]See text.

[b]In grams per 100 g dry matter.

TABLE II

Animal Performances

	NL[a] 18C	InL[b] 18C	NL 10C	InL 10C
Initial mean weight[c]	12.1	11.8	12.0	12.0
Final mean weight	25.0	26.0	27.8	28.1
Feed intake per fish[c]	19.5	21.6	21.0	19.0
Feed conversion ratio[d]	1.51	1.52	1.33	1.18

[a]NL = normal lard diet.

[b]InL = interesterified lard diet.

[c]In grams.

[d]Food intake/growth of each animal.

our study.

The specificity of action of pancreatic lipase on ester linkages at the α-position of glycerol is now well established (8,9). The chain specificity of pig lipase involves chain length and the position and number of double bonds (10). In our studies, the effect on the lipolysis rate of 1,2, or 3 double bonds was not detectable. We used fats free of long-chain polyunsaturated fatty acids to avoid hydrolysis-resistant fatty acids (11).

MATERIAL AND METHODS

Animals and Diets

A total of 320 rainbow trout (*Salmo gairdnerii* Rich.) with an initial mean weight of 12 g were distributed into four groups of 80. They were raised in plastic tanks with a capacity of 130 liters and supplied with fresh water. Two of the groups were kept at a temperature of 10 ± 1C and the two others at a temperature of 18 ± 1 C during the whole experiment (8 wk). The trout received a semisynthetic diet based on casein and maize starch. The triglyceride structure of the fat was the only variable. The dietary fats were either lard of normal structure (USU) (S and U are saturated and unsaturated fatty acids, respectively), or interesterified lard, exhibiting the same fatty acid composition (Tables III and IV). In order to verify the distribution of the saturated and unsaturated fatty acids in the interesterified triglycerides of lard, calculations were made according to Vander Wal's method (12). The weight percentage of each type of structure was the same (Table V) as that obtained according to the hypothesis of a "1 random, 2 random, 3 random" distribution (13). To avoid symptoms of deficiency, linolenic acid had to be added to the diet (14). To that end we established the following schedule: four meals per day with methyl linolenate added only to the morning meal, which was equal to one-third of the voluntary feed intake level recorded during the three previous meals. The composition of the two diets with and without methyl linolenate is given in Table I. The diet containing linolenic acid was prepared beforehand and was stored at -80 C to avoid oxidation. The feed intake level of the animals is indicated in Table II. We verified that the effective consumption of the diets with and without methyl linolenate corres-

TABLE III

Molar Fatty Acid Composition of Triacylglycerol and β-monoacylglycerol of Diet and Perigastric Adipose Tissue (P.A.T.) – Interesterified Lard

	Diet		P.A.T. 10C		P.A.T. 18C	
Fatty acid	Triglyceride	β-acylglycerol	Triglyceride	β-acylglycerol	Triglyceride	β-acylglycerol
14:0	1.3	2.8	2.7	1.4	2.6	1.5
16:0	23.9	25.1	20.9	8.6	20.8	8.0
18:0	12.1	11.7	6.0	1.2	6.2	2.0
Σ saturated	37.3	39.6	29.6	11.2	29.6	11.5
16:1ω7	2.7	2.0	10.2	15.8	8.4	13.8
18:1ω9	51.9	52.1	49.5	69.2	50.4	62.4
18:2ω6	6.2	6.3	7.4	3.9	6.8	8.8
18:3ω3	---	---	1.7	---	2.0	3.0
20:1ω9	1.9	---	1.6	---	2.8	0.5
Σ Unsaturated	62.7	60.4	70.4	88.9	70.4	88.5

ponded to the calculated consumption. The concentrate of methyl linolenate was prepared from the methyl esters of linseed oil on batch distillation and silicic acid-celite chromatography. The preparation contained 20% of 18:2ω6 and 80% of 18:3ω3.

The trout were killed by immersion in a highly concentrated bath of anaesthetic (MS 222), wiped dry, and weighed. The P.A.T. was carefully cut out and weighed. Thereafter, it was frozen in liquid nitrogen and stored at -80 C.

Analytical Methods

Analysis of Fatty acids: The analysis of fatty acids has been reported in detail previously (15) and use of a Carbowax 20M-AT phase has been discussed elsewhere (16). The samples of P.A.T. were treated directly with a chloroform-methanol mixture (2:1, v/v). The extracted lipids were cold-saponified during the night in an excess of 10% alcoholic potassium hydroxide. The unsaponifiable fraction was extracted into hexane, the fatty acids were extracted after hydrochloric acidification of the aqueous phase and methylated in a 3% methanol-HCl mixture. The methyl esters obtained were extracted with hexane and stored at -80 C before the analysis. All operations were performed under an atmosphere of nitrogen. We used an open-tubular column of stainless steel (0.56 mm, inside diameter, x 100 m), packed with Carbowax 20M + terephthalic acid, allowing reduction of 18:2ω6 and 18:3ω3 losses during the chromatography (16).

Pig pancreatic lipase: The pancreatic juice was obtained from pigs of 40-50 kg fitted with a pancreatic fistula (17). The pancreatic juice was collected in cooled containers (+2 C), lyophilized, and stored at -30 C.

Lipolysis of triglyceride by pig lipase: The digestion mixture contained 0.9M Tris-HCl buffer (pH 8.0), 1.5 mM $CaCl_2$, and 150-200 mg triglycerides in a total volume of 10 ml. The mixture did not include any bile salts in order to avoid the possible hydrolysis by nonspecific esterase (18-20). Hydrolysis was performed with 200 lipase units (microequivalents fatty acid x min^{-1}) (about 200 mg lyophilized pig pancreatic juice) and stopped after one-third of the reaction (about 20 min) by means of HCl (1/2). The reaction products were extracted with ethyl ether separated by chromatography on a defatted Kiesselgel layer by means of a hexane-ethyl ether-formic acid mixture (120:60:1.5, v/v/v) and the fatty acids of the monoglycerides were transmethylated for gas chromatographic analysis.

RESULTS

Fatty Acid Composition

Tables III and IV indicate the molar fatty acid percentages of the P.A.T. for the two temperatures and the two diets. No long-chain polyunsaturated fatty acids occurred in the tissue. The amounts of fatty acids were similar in the tissue and in the diets. However, 16:1ω7 increased in the P.A.T., whereas 16:0 decreased. There were only very small differences between the fatty acid composition of the P.A.T., from the different groups. Consequently, the fatty acid composition of the dietary triglycerides highly affected the nature and amount of fatty acids of P.A.T. triglycerides, whereas the influences of the dietary glyceride structure and of the environmental temperature were not detectable in the animals.

The Composition of the Fatty Acids of the β-position on the Triglycerides

The data obtained with the interesterified lard

TABLE IV

Molar Fatty Acid Composition of Triacylglycerol and β-monoacylglycerol of Diet and Perigastric Adipose Tissue (P.A.T.)–Normal Lard

	Diet		P.A.T. 10C		P.A.T. 18C	
Fatty acid	Triglyceride	β-acylglycerol	Triglyceride	β-acylglycerol	Triglyceride	β-acylglycerol
14:0	1.3	3.6	2.8	3.0	2.9	4.3
16:0	23.9	69.3	21.5	14.4	20.9	29.2
18:0	12.1	3.5	6.7	1.7	6.1	9.5
Σ saturated	37.3	76.4	31.0	19.1	29.9	43.0
16:1ω7	2.7	4.2	9.2	16.9	8.6	6.0
18:1ω9	51.9	17.0	47.5	58.2	48.2	43.0
18:2ω6	6.2	2.4	8.1	5.8	8.2	4.1
18:3ω3	—	—	2.0	—	2.7	0.6
20:1ω9	1.9	—	2.2	—	2.4	3.3
Σ Unsaturated	62.7	23.6	69.0	80.9	70.1	57.0

TABLE V

Experimental and Theoretical Structure of the Interesterified Lard (Weight Percentage of Each Structure)

Structure[a]	Experimental	Theoretical "at random"
S_3	5.9	5.9
S_2U	27.9	27.9
SUS	8.4	9.3
USS + SSU	19.5	18.6
SU_2	43.6	43.5
USU	15.9	14.5
UUS + SUU	27.7	29.0
U_3	22.6	22.7

[a]S and U are saturated and unsaturated, respectively.

diet (InL) in Table III indicate that trout preferentially place the U in the β-position and the S in the α-position of glycerol. Exceptions exist for U. Table VI shows more precisely that 18:2ω6 and 18:3ω3 tend to occupy the β-position at 18 C but not at 10 C. Nevertheless, the distribution pattern of the bulk of the fatty acids is identical at the two acclimatization temperatures in the animals fed InL diet.

Because of the special triglyceride structure in lard, results obtained with the normal lard diet (NL) are of particular interest. Table IV shows that at 10 C the percentage composition of the β-acyl groups from the NL diet and from the trout fed NL diet are different, whereas the compositions are rather similar in the trout fed NL diet and InL diet. However, Table VI shows that the percentage of S does not decrease to 13% as with the InL diet, but to 21%, and that the percentage of U does not increase to 42%, but to 39%, even if these two last ones are probably not significantly different. On the other hand, Table VI indicates that the difference between the P.A.T. at 10 C and the diet is increased in trout fed NL diet when compared to the trout fed InL diet: the calculated differences are 69% against 63% for S, 67% against 30% for U. This obvious tendency to synthesize triglycerides with central U and external S strongly suggests that the particular glyceride structure of the normal lard ingested does not control the β-acylation of the U in the trout.

Tables III and IV show that, at 18 C, the β-acyl groups of trout fed NL diet do not present the same fatty acid composition as the NL diet, on one hand, and the fish fed InL diet, on the other hand. It is still clear that they are subject to a modification of composition leading to increases of U whereas the S decreases. However, at 18 C, the redistribution of fatty acids from the diet is not so important as at 10 C. In the data of Table VI, we notice that the calculated difference between P.A.T. and diet is only 29% for S at 18 C, against 69% at 10 C, and only 52% for U at 18 C, against 67% at 10 C. Nevertheless, the tendency to synthesize a particular structure of triglyceride does still exist at 18 C.

TABLE VI

Fatty Acids in β-position of the Triglyceride Compared with the Fatty Acid Composition of the Triglyceride

Fatty acid	Interesterified lard			Normal lard		
	Diet	P.A.T.[a] 10C	P.A.T. 18C	Diet	P.A.T. 10C	P.A.T. 18C
14:0	72[b]	17	19	92	36	49
16:0	35	14	13	97	22	47
18:0	32	7	11	10	8	52
Σ saturated	35	13	13	68	21	48
16:1ω7	25	52	55	52	61	23
18:1ω9	33	47	41	11	41	30
18:2ω6	34	18	43	13	24	17
18:3ω3	---	---	50	---	---	7
20:1ω9	---	---	6	---	---	46
Σ Unsaturated	32	42	42	13	39	27

[a]P.A.T. = perigastric adipose tissue.

[b]Moles of the fatty acid in β-position for 100 moles of the same fatty acid in the triglyceride.

It should also be noted that the 18:3ω3 and 20:1ω9, and to a minor extent the 18:2ω6, do not occupy the same position as the other more abundant unsaturated fatty acids. In particular, it is apparent from Table VI that 18:2ω6, 18:3ω3, and 20:1ω9 prefer the α-position in general (percentages <33%). On the other hand, exceptions to be noted at 18 C are: 18:2ω6 and 18:3ω3 in trout fed InL diet, and 20:1ω9 in trout fed NL diet.

DISCUSSION

The acylation of the three hydroxyl groups of glycerol is not achieved evenly in the trout. It is governed by the type of fatty acids involved. The saturated fatty acids are acylated in the α-position, whereas the bulk of unsaturated fatty acids is acylated in the β-position. It is very apparent that the glyceride structure in the trout, to a large extent, does not depend on the dietary glyceride structure. A USU type structure cannot prevent the rebuilding of the specific structure of trout. There is no retention of the β-monoglyceride structure in the triglyceride metabolism. These observations and other results concerning the specificity of action of the pancreatic lipase in the trout (21) suggest that the β-monoglyceride lipase may be a rather important enzyme in the glyceride catabolism, and that the biosynthetic pathway through the L-α-glycerophosphate is probably preponderant in synthesizing triglycerides.

It is clear from the results obtained at 18 C, that the fish cannot completely modify the distribution of fatty acids of diet. The hypothesis of an enzymatic saturation in the enterocyte agrees with observations concerning the lipid overloading of the intestinal cell (22,23) in the trout, and recent observations according to which overloading probably occurs rather at 18 C than at 10 C (Unpublished data). Further research is required to confirm the results obtained in Table II. Is the glyceride structure an important factor for obtaining the best feed conversion ratio? Preferential acylation of 18:2ω6, 18:3ω3, and 20:1ω9 is dependent on the acclimatation temperature, and may be important with regard to the fundamental physiological consequences of this phenomenon on the cell structures and on the environmental conditions of enzymatic systems.

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Non-Methylene-Interrupted and ω4 Dienoic Fatty Acids of the White Shrimp *Penaeus setiferus*

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ABSTRACT

The total lipid fatty acids from the white shrimp *Penaeus setiferus* were found to contain several unusual dienoic fatty acid species. These included two methylene-interrupted species: Δ11,14-$C_{18:2}$ (18:2ω4) and Δ13,16-$C_{20:2}$ (20:2ω4). Also found were several non-methylene-interrupted dienoic fatty acids including Δ7,11 and Δ7,13-$C_{20:2}$, Δ7,13-$C_{21:2}$, Δ7,13, Δ7,15, Δ9,13, Δ9,15, and Δ7,17-$C_{22:2}$. Many minor $C_{20:2}$ non-methylene-interrupted dienes were found but could not be unequivocally characterized.

INTRODUCTION

Non-methylene-interrupted dienoic (NMID) fatty acids have been found in a number of plants and animals (1-11). Open tubular gas chromatography of the total lipid fatty acid methyl esters from the white shrimp (*Penaeus setiferus*) revealed several C_{20}, C_{21}, and C_{22} fatty acids, which had retention times similar to those found for NMID fatty acid methyl esters of marine origin (9,10). Due to the commercial significance of *P. setiferus* as a food source and a possible physiological role for these unusual fatty acids, we pursued their structural elucidation.

EXPERIMENTAL PROCEDURES

White shrimp were netted in the Intracoastal waterway at Dewees Island, South Carolina in mid-August, 1975. Live specimens were transported to the laboratory where they were stored at -20 C for 2 wk until extraction. The shrimp lipids were extracted by the method of Bligh and Dyer (12), and fatty acid methyl esters were prepared by heating the lipids in 3% methanolic H_2SO_4 in sealed tubes under an atmosphere of nitrogen at 60 C for 2 hr. The fatty acid methyl esters were purified by thin layer chromatography (TLC) on silica gel (Supelcosil 12A, Supelco, Inc., Bellefonte, PA.) by developing in chloroform. The TLC plate was sprayed with 0.1% ethanolic 2',7'-dichlorofluorescein (Fisher Scientific Co., Atlanta, GA.), and the lipid bands were visualized under ultraviolet light. The fatty acid methyl ester band was extracted from the adsorbent in chloroform-methanol-water (50:50:1). The solvent was evaporated to dryness, and the fatty acid methyl esters were taken up in hexane.

The fatty acid methyl esters were analyzed on a Hewlett-Packard 5711A gas chromatograph fitted with a 45.7 m x .25 mm ID open-tubular stainless steel column coated with Silar-5CP (Perkin-Elmer Corp., Inc., Norwalk, CT). Gas chromatographic temperature conditions were as follows: injector 300 C, column 170 C, detector 300 C. Helium carrier gas pressure was 50 psig. The fatty acid methyl esters were separated according to degree of unsaturation on TLC plates coated with silica gel (Supelcosil 12A) impregnated with 5% $AgNO_3$. The plates were sequentially developed three times in chloroform at 5 C, sprayed with 0.1% ethanolic 2',7'-dichlorofluorescein and were visualized under ultraviolet light. The dienoic fatty acid methyl esters were resolved into 3 bands. Each of these bands was scraped off the plate and extracted with chloroform-methanol-water (50:50:1). The solvent was evaporated with a stream of nitrogen, and the methyl esters were taken up in hexane. Each of the three diene bands was analyzed under the gas chromatographic conditions stated above. One portion of each band was also hydrogenated and analyzed by gas chromatography. Another portion was derivatized to the tetra-0-trimethylsilyl (TMS) derivatives of the fatty acid methyl esters after treatment with OsO_4 (13). Pyrrolidide derivatives were also prepared (14,15). The tetra-0-TMS derivatives of the upper and middle TLC bands were analyzed on a Finnigan 3200 gas chromatograph-mass spectrometer (GC-MS) fitted with a 2 mm x 2 m glass column packed with 2% OV-17 on 100/120 mesh Supelcosil AW-DMCS. The accelerating voltage was 70 eV. The tetra-0-TMS derivatives of the lower TLC band were analyzed on an LKB 9000 GC-MS fitted with a 2 mm x 1.1 m glass column packed with 3% SP-2100-DA on 100/120 mesh Supelcon AW-DMCS. The accelerating voltage was 22.4 eV. Pyrrolidide derivatives were analyzed on an LKB 9000 GC-MS at 70 eV as described above. For GC-MS, several

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temperature programs were used in each case, but were usually 150-280 C at 5-10 C/min.

NMID species were separated on the basis of chain length by preparative gas chromatography on a Perkin-Elmer 801 gas chromatograph utilizing a 4 mm x 7 m glass column packed with 10% Apeizon-L on 100/120 mesh Supelcoport. Gas chromatographic conditions were: injector 290 C, column 280 C, detector 300 C. Reductive ozonolysis was carried out on the C_{20} and C_{22} chain lengths with a Supelco Micro Ozonizer (Supelco, Inc., Bellefonte, PA). Ozonides were prepared in 200-500 μl of methylene chloride and cleaved into aldehyde and aldehydic ester products by the addition of about 1 mg of triphenylphosphine (16,17). These products were chromatographed on a Beckman GC-45 gas chromatograph utilizing a 4 mm x 2 m glass column packed with 8% OV-101 on 100/120 mesh Supelcoport. Oven temperature was programmed from 50 C to 250 C under the following gas chromatographic conditions: injector 280 C, detector 300 C, and He carrier gas flow rate 40 ml/min. Flow rates for the flame ionization detector were: air 300 ml/min, He makeup 50 ml/min, and H_2 50 ml/min.

RESULTS AND DISCUSSION

Initially gas chromatography of the total lipid fatty acid methyl esters revealed the probable presence of a small quantity of C_{22} NMID fatty acids in *P. setiferus* according to their retention times (9,10). They comprised about 0.1% by weight of the total fatty acids. TLC of the fatty acid methyl esters on siliver nitrate-impregnated silica gel resolved the dienoates into three bands. Gas chromatography of the dienoates recovered from each band showed that the uppermost TLC band probably contained 20:2ω6 (ECL 20.77) and a minor component (ECL 20.97) as shown in Figure 1A. A similar pattern was observed upon analysis of the middle TLC band which was mainly 18:2ω6 (ECL 18.77), also followed by a minor component with an ECL of 18.97 (Fig. 1B). The lower band contained components tentatively identified as NMID fatty acid methyl esters (Fig. 1C). Hydrogenation of all the recovered dienoates resulted in their conversion to straight-chain saturated methyl esters, eliminating the possibility of branched structures.

Mass spectra of the tetra-0-TMS derivatives of the fatty acid methyl esters recovered from the top and middle TLC bands were typical of these derivatives and clearly showed that the major components were 20:2ω6 and 18:2ω6,

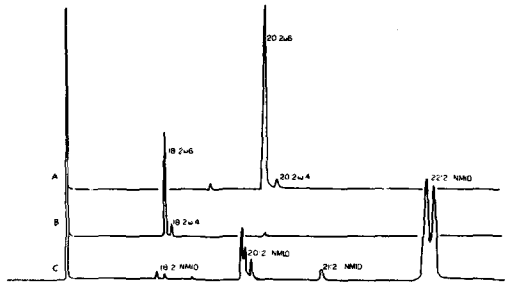

FIG. 1. Open-tubular gas chromatograms of dienoic fatty acid methyl ester fractions isolated by thin layer chromatography on $AgNO_3$-impregnated silica gel. The dienoic fatty acid methyl esters were well resolved by TLC according to chain length and fatty acid type. The top TLC band (A) and the middle TLC band (B) contained the methylene-interrupted $C_{20:2}$ and $C_{18:2}$ species. The lower TLC band (C) contained the non-methylene-interrupted dienoic (NMID) fatty acid methyl esters.

as expected. Under the gas chromatographic conditions used, the minor components were not completely resolved from the ω6 isomers, but mass spectra obtained from the shoulder on the tailing edge of the major gas chromatographic peaks clearly showed the presence of intense ions at m/e 145 (ω4) and 287 (Δ11) for the C_{18} isomers and at m/e 145 and 315 (Δ13) for the C_{20} isomer. These fatty acid species were, therefore, identified as 18:2ω4 and 20:2ω4.

Tentative identification of 18:2ω4 and 20:2ω4 in the Atlantic sturgeon *(Acipenser oxyrhynchus)* has recently been reported (18). Our study confirms these structures by mass spectrometry. These ω4 dienoates are probably synthesized by chain elongation of algal 16:2ω4, since this fatty acid occurs in most marine species. We did not, however, detect 16:2ω4 in all of the white shrimp lipid samples after TLC of the fatty acid methyl esters on silver nitrate impregnated silica gel. When detected, 16:2ω4 was always found in the NMID fraction. Although less plausible, an alternate biosynthetic pathway would involve a partial lack of specificity of the desaturase system utilized by plants for the synthesis of 18:2ω6 from 18:1ω9. In this case, 18:1ω7 would be desaturated to 18:2ω4 which would be chain elongated to 20:2ω4. It is noteworthy that because of the retention times of 16:2ω4, 18:2ω4, and 20:2ω4 (ECL 16.95, 18.97, and 20.97), these three components could easily be confused with 17:0, 19:0, and 21:0 on the Silar-5CP column used.

Analysis of the components recovered from the lowest TLC diene band by open-tubular gas chromatography showed that the NMID chain

TABLE I

Comparison of (A) Equivalent Chain Lengths (ECL) of Non-Methylene-Interrupted Dienoic (NMID) Isomers and their Relative Percentage Composition as Determined by Gas Chromatography[a], and (B) NMID Isomers Identified and their Percentage Composition as Determined by Mass Fragmentography[b]

A		B	
ECL	% by wt	Isomer	% by wt
C_{20} NMID isomers			
20.48	10.1		
20.52	6.0	Δ7,13	8
20.63	3.9	Others	2
	C_{20} total 20.0		C_{20} total 10
C_{21} NMID isomers			
21.58	2.5	Δ7,13	2
C_{22} NMID isomers			
22.52	47.2	Δ7,13	49
22.55	30.3	Δ7,15	25
	C_{22} total 77.5	Δ9,13	10
		Δ9,15	2
		Δ7,17	2
			C_{22} total 88

[a]ECL values and percentages were determined on Silar-5CP as described in text. Relative percentages of very minor components (ECL 18.56, 18.83, and 19.52) were not calculated.

[b]Relative percentages of isomers were calculated from peak areas of characteristic ions as described in experimental procedures.

TABLE II

Reductive Ozonolysis Products

Chain length of isolated isomers	Aldehydes	Mole %	Aldehyde esters	Mole %
C_{20}	C_7	25.7	C_5	21.7
	C_9	74.3	C_7	78.3
C_{22}	C_7	38.8	C_7	100.0
	C_9	61.2		

lengths were represented by C_{18}, C_{19}, C_{20}, C_{21}, and C_{22} species as shown in Figure 1C. The ECL values and relative percentages of the NMID species, as determined by gas chromatography on Silar-5CP, are listed in Table IA. Using conventional plotting procedures (19), we found that the C_{20} NMID isomers did not plot with the C_{22} NMID isomers. It was, therefore, concluded that the major isomers within these two chain lengths were not homologous.

The C_{20} and C_{22} NMID species were isolated on the basis of chain length by preparative gas chromatography, and each was submitted to reductive ozonolysis (16,17). Insufficient amounts of other chain lengths were available for this analysis. Reductive ozonolysis of the C_{20} NMID isomers yielded the following fragments in order of their abundance: a C_7 aldehydic ester, C_9 aldehyde, C_7 aldehyde, and C_5 aldehydic ester, as shown in Table II. Similarly, the C_{22} NMID isomers yielded a C_7 aldehydic ester, C_9 aldehyde, and C_7 aldehyde. Ozonolysis, therefore, showed that the maior C_{20} NMID isomers consisted mainly of Δ7,11 and Δ7,13-$C_{20:2}$, with a small amount of Δ5,11 and Δ5,13 possibly present. The major C_{22} NMID isomers were identified as Δ7,13 and Δ7,15-$C_{22:2}$.

Oxidation of both chain lengths by the von Rudloff method (20) yielded heptanedioic acid as the major cleavage product, but large numbers of minor monocarboxylic and dicarboxylic acids were also observed, indicating that excessive oxidation had probably occurred. Although individual isomers could not be identified with this procedure, the predominance of a Δ7 double bond in both chain lengths was confirmed.

Conversion of the NMID fatty acid methyl esters to pyrrolidide derivatives followed by

GC-MS (14,15) confirmed that the C_{18}, C_{20}, C_{21}, and C_{22} species were dienoic fatty acids as shown by discrete molecular ions at m/e 333, 361, 375, and 389. Double bond positions could not be deduced from pyrrolidide mass spectra because of the presence of isomers within each gas chromatographic peak.

Mass spectra of the tetra-0-TMS derivatives of the NMID fatty acid methyl esters were typical (11). The total ion intensity resulting from GC-MS (mass fragmentography) of these derivatives is shown in Figure 2A. Single peaks were essentially obtained from the C_{20} and C_{21} chain lengths, but the C_{22} isomers were fairly well resolved into three peaks.

Figure 2B contains ion plots of m/e 231 and 259, representing diagnostic ions for double bonds in the Δ7 and Δ9 positions. Similarly, plots of the ions m/e 187, 201, and 215 (ω7, ω8, and ω9) are shown in Figure 2C. Each relative peak area represented by diagnostic ions was digitally integrated, and these areas were used to calculate estimated relative abundances (Table IB) of NMID fatty acid species in the sample.

In Figures 2B and 2C, the major C_{20} species identified by GC-MS was Δ7,13-$C_{20:2}$ (m/e 231 and 187), representing about 8% of the NMID fatty acids in this sample. As shown in Figure 1C, at least two other C_{20} isomers were present, but these were not as evident in the sample analyzed by GC-MS, and we were unable to determine their structures by GC-MS. In Figure 2A, a shoulder is clearly present on the proximal side of the total ion intensity plot of the C_{20} isomers. It comprised about 2% of the NMID fatty acids and was largely accounted for by the ion m/e 187 (ω7) as shown in Figure 2C. Based on gas chromatographic considerations alone, we thought that this might represent the C_{20} NMID species containing a Δ5 double bond, but no single Δ ion found in this region was abundant enough to account for the area in this shoulder. Although the ion plot of m/e 203 (Δ5) showed that it was present in this region, the ion plot was virtually flat when converted to the scale used in Figure 2 and is therefore not shown. The ions at m/e 159(ω5), 173(ω6), 215(ω9), 245(Δ8), and 259(Δ9) were also clearly present in mass spectra taken in the C_{20} region, but combined they represented a very small percentage of the total. No attempt was made to identify and quantitate the large number of possible isomers.

The only key ions observed in the C_{21} region were m/e 231 (Δ7) and m/e 201(ω8), establishing this species as Δ7,13-$C_{21:2}$ which accounted for about 2% of the NMID fatty acids.

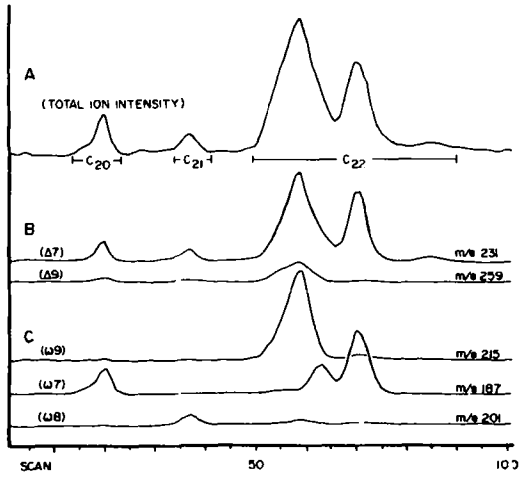

FIG. 2. Total ion intensity and plots of major diagnostic ions obtained from tetra-0-TMS derivatives of shrimp non-methylene-interrupted dienoic (NMID) fatty acids. Plots of ions which were diagnostic of double bond positions only are shown. A, Total ion intensity. B, Ion plots of m/e 231 and 259. C, Ion plots of m/e 187, 201, and 215.

Five C_{22} NMID species were identified. The most abundant Δ ions in C_{22} region were m/e 231(Δ7) and 259(Δ9) as shown in Figure 2B. In the C_{22} region, these ion plots clearly show m/e 231 split into three peaks and m/e 259 present as only one peak. The most abundant ω ions were m/e 215 (ω9) and 187 (ω7) as shown in Figure 2C. The ion m/e 215 was confined to one peak while m/e 187 was split into two peaks. The predominant C_{22} NMID was therefore Δ7,13-$C_{22:2}$, which comprised about 49% of the NMID species (Table IB). This was followed in relative abundance by Δ7,15-$C_{22:2}$ (25%), Δ9,13-$C_{22:2}$ (10%), and Δ9,15-$C_{22:2}$ (2%), according to calculations of relative ion abundances. It is noteworthy that although the tetra-0-TMS derivative of Δ9,15-$C_{22:2}$ followed the derivative of Δ9,13-$C_{22:2}$ as expected, both cochromatographed with the derivative of Δ7,13-$C_{22:2}$. According to expected gas chromatographic behavior, the Δ9 species should have followed the Δ7 species (Fig. 2). A small amount (2%) of Δ7,17-$C_{22:2}$ was present in the last peak as was shown by ions at m/e 231 (Fig. 2B) and m/e 159 (not shown in Fig. 2).

In Table III, it may be seen that there was general agreement between the data obtained by all three analytical methods. The mole percentages obtained by gas chromatography and by reductive ozonolysis correspond very closely, but the analysis of the tetra-0-TMS derivatives by GC-MS yielded results which did not entirely agree with the others. In Table IA, the

TABLE III

Summary of Analysis of Shrimp Non-Methylene-Interrupted Dienoic (NMID) Fatty Acids According to Chain Length by Gas Chromatography, Reductive Ozonolysis, and Mass Fragmentography

Isomer	Gas Chromatography (mole %)	Reductive Ozonolysis (mole %)	Mass Fragmentography (mole %)
C_{20}			
Δ 7,11	50.5	51.2	
Δ 7,13	30.0	27.1	80
Δ 5,11 + Δ5,13		21.7	
unidentified	19.5	---	20
C_{22}			
Δ 7,13	60.9	61.2	56
Δ 7,15	39.1	38.8	29
Δ 9,13	---	---	11
Δ 9,15	---	---	2
Δ 7,17	---	---	2

three C_{20} NMID isomers comprised 20% of the NMID methyl esters as determined by gas chromatography, but according to mass spectrometric data (Table IB), the C_{20} isomers comprised only 10% and were represented almost entirely by Δ7,13-$C_{20:2}$. Since gas chromatography revealed three C_{20} isomers and reductive ozonolysis showed the major C_{20} isomer to be Δ7,11, a selective loss of this and other C_{20} isomers probably occurred, either as a result of derivatization or GC-MS. The GC-MS analysis also showed that the total relative percentage of C_{22} NMID species was 88% (Table IB), which is about 11% higher than the figure obtained by gas chromatographic analysis (Table IA). The difference is almost completely accounted for by the diminished total ion intensity area (10%) obtained for the C_{20} isomers. Although this shows that the greatest difference between analysis by GC-MS and the other methods was attributable mainly to the loss of the Δ7,11-$C_{20:2}$ isomer, it seems clear also that the relative amounts of C_{22} isomers containing a Δ9 double bond were exaggerated. Only trace amounts of C_9 aldehydic ester were detected when the C_{22} isomers were analyzed by reductive ozonolysis.

The origin of NMID fatty acids is not known. The C_{18} NMID species in human milk contain double bonds in the Δ8,12, Δ9,13, Δ9,15, Δ9,16, and Δ11,15 positions (2). The predominant NMID species in beef and mutton fat is Δ11,15-$C_{18:2}$ (1). Lipids from oyster *(Crassostrea virginica)* were shown to contain Δ5,11-$C_{20:2}$, Δ5,13-$C_{20:2}$, Δ7,13-$C_{22:2}$, and Δ7,15-$C_{22:2}$ as the major NMID components by reductive and by oxidative ozonolysis (10). The periwinkle *(Littorina littorea)*, moon snail *(Lunatia triseriata)*, sand shrimp *(Crangon septemspinosus)*, and the atlantic sturgeon *(Acipenser oxyrhynchus)* are thought also to contain the same major NMID species as the oyster, according to gas chromatographic retention data (9). The sand shrimp contained about the same concentration of NMID fatty acids that we found in the white shrimp. These unusual fatty acids were not reported in other studies of shrimp fatty acids (21,22); however, the analytical methods used in those studies would not have resolved NMID species from the $C_{20:1}$ and $C_{22:1}$ fatty acid methyl esters. We have tentatively shown the presence of C_{22} NMID fatty acids in three other crustacean species including the mantis shrimp *Squilla empusa,* brown shrimp *P. aztecus,* and the blue crab *Callinectes sapidus.* According to gas chromatographic retention behavior, these fatty acids appear to be the same C_{22} NMID species identified in this study.

In our study of *P. setiferus*, the major C_{20}, C_{21}, and C_{22} NMID species all contained a Δ7 double bond. The Δ7 NMID species comprised at least 90% of the NMID fatty acids. With the Δ7 double bond as a relatively constant feature individual NMID species of a given chain length were, therefore, determined, for the most part, by the position of the double bond nearest the terminal methyl end. This was unexpected since the biosynthesis of the major shrimp NMID fatty acid (Δ7,13-$C_{22:2}$) by chain elongation, for example, would require a Δ5,11-$C_{20:2}$ precursor and only small relative amounts of the latter were present. This infers that if NMID fatty acids are synthesized by shrimp, the biosynthesis of each NMID chain length probably occurs by desaturation of the corresponding monoenoate species of the same chain length which already contains a double bond distal to the Δ7 position (9). The finding of the Δ7 double bond in the odd carbon

$C_{21:2}$ NMID species lends strength to this concept. Since the NMID fatty acids are minor components of white shrimp lipids, it is quite possible that they are of exogenous origin.

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Glyceride Metabolism in the Myopathic Hamster

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ABSTRACT

Analysis of plasma lipids of 30- and 185-day-old BIO 82.62 myopathic hamsters and age-matched normal controls revealed a decrease in only the concentration of cholesteryl esters of 185-day-old diseased animals. Measurement of lipoprotein lipase (LPL) activity in heart, muscle, and adipose tissue showed no difference between the activity of the enzyme in the heart and muscle of the cardiomyopathic hamsters and that of the age-matched controls. In adipose tissue, however, LPL activity was depressed in the diseased animals in both age groups. No difference was found in the activity of hormone sensitive lipase. Incorporation of sn[U-^{14}C] glycerol-3-phosphate into total lipids was found to be depressed in homogenates of heart, muscle, and adipose tissue but unchanged in liver homogenates of diseased animals. It was concluded that the decrease in the capacity to synthesize glycerides, rather than limiting substrate concentrations, could be the cause of the decrease in the lipid content in some tissues of the cardiomyopathic hamster.

INTRODUCTION

In a previous study of the lipid content of heart and muscle of cardiomyopathic and age-matched normal hamsters, we reported (1) a decrease in the total lipid, triglyceride, and cholesterol content of heart and an increase in the cholesterol content of muscle. These variations in the lipid content could be a result of alterations in lipid metabolism in these and other organs. Decreased synthesis, increased degradation, limited uptake of circulating lipids and/or depression in the concentration of circulating lipids could cause the observed variations. Evidence of depression in fatty acid oxidation in homogenates of cardiac muscle of cardiomyopathic hamsters has been reported (2,3). Recent findings in our laboratory, on the other hand, have revealed a significant decrease in the activities of several of the enzymes of de novo fatty acid synthesis in the liver and adipose tissue of the diseased animals (4). These results led us to hypothesize that the depression in lipogenesis might limit glyceride synthesis if the concentration of fatty acids became limiting.

Although the aforementioned findings would be consistent with the observed alternations in the lipid content of heart and muscle, more direct evidence was necessary. Accordingly the present study was undertaken to compare cardiomyopathic and normal hamsters with regard to the concentration of circulating lipids, the ability of adipose tissue in the same animals to mobilize depot fat, the capacity of various tissues to take up circulating lipids, and the ability of tissue homogenates to synthesize glycerides.

METHODS

Animals

Randomly bred normal and BIO 82.62 cardiomyopathic hamsters were purchased from TELACO (Trenton Experimental Laboratory Animals Company, Bar Harbor, ME) when they were ca. 25-days-old. They were reared in our facilities under identical conditions. At an average age of 33 days (span 31-35 days) and 185 days (span 180-190 days), at least six animals of the BIO 82.62 strain and an equal number of age-matched normal controls were sacrificed by decapitation. After being excised and suspended in ice cold 1.15% KCl, heart, muscle, and liver were cleaned from adhering fat and connective tissue, blotted dry, and weighed. All tissue preparations were used fresh. Protein concentration of liver and muscle homogenates was determined by the biuret method (5) and that of heart and adipose tissue by the Lowry method (6).

Plasma Lipid Analysis

Hamster blood was obtained by cardiac puncture of ten nembutal-treated animals of each strain (50 mg/kg weight). Plasma lipids were extracted according to the method of Bligh and Dyer (7), an aliquot taken for total lipid determination by the acid-dichromate method (8), and separated by thin layer chromatography (TLC) as previously described (9). The triglyceride and free fatty acid contents were determined by the acid-dichromate

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method (8), cholesteryl esters and free cholesterol by the sulfophosphovanillin reaction (10) as modified by Tapscott and Dohm (11), and the phospholipid phosphorous by the method of Bartlett (12) as modified by Kankare and Souvaniemi (13).

Assay of Tissue Lipases

Epinephrine-stimulated lipase (ESL) activity was assayed by the method of Ho et al. (14) as modified by Askew et al. (15). Subcutaneous fat pads were removed, rinsed in 1.15% KCl, and incubated with gentle shaking for 1 hr at 37 C, pH 7.3, in a Krebs-Henseleit (16) buffer containing 5% fat-free bovine serum albumin. Tissue to buffer ratio was 1:5 (w/v) and L-epinephrine was present at 20 μg/ml. One-half of a fat pad was used for the assay of epinephrine-stimulated lipase activity while the other half served as a nonepinephrine control. The other fat pad was used as a duplicate. Following incubation, a 1 ml aliquot of the buffer surrounding the fat pad was removed and the free fatty acids (FFA) extracted and titrated according to the method of Dole and Meinertz (17).

For the assay of lipoprotein lipase (LPL) activity, the tissues were homogenized in 9 volumes of Chapell-Perry buffer (18), pH 7.4. Homogenates were centrifuged at 600 x g for 10 min and 0.2-0.5 ml of the supernatant solution was used for the assay of LPL activity. LPL activity was determined, in duplicate, according to the method of Korn (19) using serum-activated Ediol (Sigma Chem. Co., St. Louis, MO) as a substrate. Following a 60 min incubation period, the reaction was terminated and FFA were extracted and titrated as described above. All reactions were linear with respect to enzyme concentration and incubation time.

Glyceride Synthesis

The capacity of homogenates of hamster tissue to synthesize glycerides was estimated by a modification of the method of Askew et al. (20). Hamster heart, skeletal muscle, and adipose tissue were homogenized in 5 volumes of 10 mM phosphate buffer, pH 7.4 containing 1 mM EDTA. Livers were homogenized in 5 volumes of 0.05 M Tris buffer, 0.1 M KCl, 5 mM $MgSO_4$, and 1 mM EDTA, pH 7.5. The cofactor concentrations of the incubation mixtures and assay conditions are shown in Table I. Under those conditions, reactions were linear with respect to enzyme concentration and incubation time.

All reactions, which were run in duplicate, were terminated by the addition of 3.0 ml of chloroform-methanol (1:2) after 15 min of incubation, and the lipids were extracted by the Bligh and Dyer method (7). One-half ml of the lower phase was withdrawn, evaporated to dryness, and counted in a scintillation medium containing 6% PPO in 20% (v/v) methanol and 80% (v/v) toluene. Another 1 ml of the lower phase was evaporated to dryness and redissolved in a minimal volume of chloroform-methanol (2:1). Neutral glycerides were separated by TLC on Adsorbosil-5 (Applied Science) as described previously (1), and the spots corresponding to neutral glycerides on the TLC plates were scraped into counting vials and counted in the same scintillation solution mentioned above. The recovery of triglycerides through this procedure was about 100%.

TABLE I

Assay Conditions for sn[U-^{14}C] glycerol-3-Phosphate (G3P) Esterification by Tissue Homogenates[a]

Cofactor	Heart	Muscle	Liver	Adipose tissue
	mM Concentration			
ATP	5.00	5.00	3.00	3.30
CoA	0.05	0.05	0.40	0.08
$MgCl_2$	5.00	5.00	3.00	2.00
NaF	20.00	20.00	–	6.70
K_2HPO_4	50.00	50.00	3.00	50.00
Palmitate	0.40	0.20	0.80	0.40
G3P	15.00	15.00	15.00	15.00
Homogenate (ml)	0.10	0.10	0.10	0.20

[a]All reactions were incubated for 15 min at 37 C in a final volume of 0.8 ml; sp. activity of sn[U-^{14}C] glycerol-3-phosphate was 0.01 μC/μmole. Albumin-bound palmitate was prepared according to the method of Bjorntorp (29).

RESULTS

Table II summarizes the results of the determinations of organ weights and protein concentration of each organ in mg/g tissue of 30- and 185-day-old cardiomyopathic and age-matched controls as well as body weights of 185-day-old animals of both strains. In the first age group (age 30 days), no differences between the diseased and normal hamsters in organ weights or protein concentration of these tissues were found. Adipose tissue weights were significantly lower in the 185-day-old cardiomyopathic hamsters. In the 185-day-old BIO 82.62 strain, protein concentration was significantly elevated in muscle, significantly depressed in liver, and not different in heart and adipose from the normal controls. Despite the significant decrease in body weight of 185-day-old diseased hamsters, food consumption per g body weight

TABLE II

Body Weight, Organ Weights, and Protein Concentrations of 30- and 185-Day-Old Ramdomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters

	Age 30 days				Age 185 days			
	RB	BIO	RB	BIO	RB	BIO	RB	BIO
	Weight (g)		Prot. conc. (mg/g)		Weight (g)		Prot. conc. (mg/g)	
Heart	0.19 ± 0.003[a]	0.19 ± 0.008	105 ± 5	115 ± 4	0.44 ± 0.01	0.43 ± 0.02	122 ± 5	116 ± 1
Muscle	2.04 ± 0.17	2.36 ± 0.20	110 ± 9	121 ± 8	3.95 ± 0.09	3.98 ± 0.1	115 ± 9[b]	144 ± 8[b]
Liver	2.22 ± 0.18	2.24 ± 0.05	268 ± 11	257 ± 4	4.01 ± 0.29	4.23 ± 0.19	293 ± 8[c]	235 ± 23[c]
Subcutanous fat pads	0.81 ± 0.05	0.81 ± 0.02	13.5 ± 1.0	13.4 ± 0.5	2.06 ± 0.29[d]	0.67 ± 0.09[d]	10.4 ± 0.9	11.5 ± 0.7
Body[f]					120.0 ± 4.5[e]	92.8 ± 4.7[e]		

[a]Values are of $\bar{X}$ ± SEM of at least six observations.
[b-e]Values with similar superscripts are statistically significant ($P<.05$).
[f]Body weights for the 30-day-old hamsters were not determined. At that age, however, the two groups are comparable in weight: RB = 57.1 ± 2.0; BIO = 48.7 ± 2.1 (See Ref. 32).

by the 185-day BIO 82.62 animals was identical to that of the normal controls—i.e., randomly bred animals consumed 0.069 ± 0.003 g food per day per g body weight and BIO 82.62 hamsters consumed 0.069 ± 0.005 g food per day per g body weight.

The results of the analyses of plasma lipids of 30- and 185-day-old cardiomyopathic and age-matched normal controls, which were in close agreement with those reported for normal hamsters (21), are summarized in Table III. No difference was observed in the concentrations of any of the lipid classes quantitated except for the decrease in the concentration of cholesterolesters of 185-day-old diseased animals.

In Table IV are shown the results of the assays of lipoprotein lipase of hamster heart, muscle, and adipose tissue. The enzyme activities that we observed are similar to those reported by Askew et al. (22) for the same tissues of the rat, but were lower than those reported by Kelley (23) for hamster heart. The difference between our observations and Kelley's could be due to the different methods employed. No differences between the cardiomyopathic and the age-matched normal controls was observed in the enzyme activity in either heart of muscle. Only in homogenates of adipose tissue was the activity of the enzyme in the two cardiomyopathic groups severely depressed.

Table V summarizes the results of the assay of epinephrine-stimulated lipase (ESL) in adipose tissue. There was no change in either the basal level or the epinephrine-stimulated activity of the enzyme in the two groups.

The results of the incorporation of sn[U-^{14}C] glycerol-3-phosphate (G3P) into total lipids by homogenates of heart, muscle, liver, and adipose tissue, which are in close agreement with those reported for the same tissues in the rat (24), are summarized in Table VI. Incorporation of the isotope by heart and muscle homogenates of 185-day-old cardiomyopathic hamster was significantly lower ($P<.05$) than age-matched normal controls. Although a similar trend could be seen in the 30-day-old animals, the difference was not statistically significant. No differences were observed in the incorporation of the isotope by liver homogenates. In adipose tissue, incorporation of G3P was lower in the homogenates of both 30- and 185-day-old diseased animals.

Table VII shows the incorporation of G3P into neutral glycerides by homogenates of the same tissues. Incorporation of the isotope by heart homogeantes of 185-day-old cardiomyopathic hamsters was significantly lower ($P<.05$) than age-matched normal controls. In

TABLE III

Plasma Lipid Levels of 30- and 185-Day-Old Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters[a]

	Age 30 days		Age 185 days	
	RB	BIO	RB	BIO
Total lipids	3.13 ± 0.30	3.47 ± 0.14	3.70 ± 0.16	3.61 ± 0.12
Cholesterol esters	0.77 ± 0.05	0.76 ± 0.06	0.73 ± 0.02[c]	0.65 ± 0.03[c]
Phospholipids[b]	1.33 ± 0.07	1.40 ± 0.03	1.28 ± 0.03	1.24 ± 0.03
Triglycerides	0.50 ± 0.06	0.48 ± 0.05	0.34 ± 0.08	0.48 ± 0.05
Cholesterol	0.31 ± 0.02	0.36 ± 0.02	0.29 ± 0.02	0.34 ± 0.02
Fatty acids	0.21 ± 0.04	0.26 ± 0.07	0.17 ± 0.02	0.14 ± 0.02

[a]All values are expressed as mg/ml plasma. The values shown are $\bar{X}$ ± SEM of at least six observations.

[b]mg/ml Phospholipid was calculated by assuming a mol wt of 650 for total phospholipid.

[c]Values with similar superscripts are statistically significant ($P<.05$)

TABLE IV

Lipoprotein Lipase Activity[a] in Heart, Muscle, and Adipose Tissue of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters

	Age 30 days		Age 185 days	
	RB	BIO	RB	BIO
Heart	0.32 ± 0.03	0.27 ± 0.03	0.37 ± 0.05	0.41 ± 0.03
Muscle	0.13 ± 0.01	0.12 ± 0.01	0.054 ± 0.01	0.074 ± 0.01
Adipose	2.46 ± 0.18[b]	1.76 ± 0.13[b]	1.16 ± 0.07[c]	0.82 ± 0.04[c]

[a]Activity expressed as uEq FFA/hr/mg protein. Values are $\bar{X}$ ± SEM of at least six observations.

[b,c]Values with similar superscripts are statistically significant ($P<.05$).

TABLE V

Adipose Tissue Epinephrine-Stimulated Lipase (ESL) Activity[a] of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters

	Age 30 days		Age 185 days	
	RB	BIO	RB	BIO
ESL activity (+) Epinephrine	2.16 ± 0.23	2.15 ± 0.30	2.78 ± 0.31	2.76 ± 0.20
ESL activity (−) Epinephrine	1.43 ± 0.18	1.32 ± 0.22	0.61 ± 0.01	0.62 ± 0.09

[a]Activity expressed as uEq free fatty acid/hr/gm tissue. Values are $\bar{X}$ ± SEM of at least six observations.

muscle, there was an increase in neutral glyceride synthesis in homogenates of 30-day-old diseased hamsters but no difference in 185-day-old animals. An increase in glyceride synthesis was observed in 185-day-old liver homogenates of cardiomyopathic hamsters. In adipose tissue, glyceride synthesis was significantly depressed in the 30- and 185-day-old cardiomyopathic hamsters.

DISCUSSION

The results of this study indicate that impairment in de novo glyceride synthesis, rather than limiting substrate concentration, could cause the decrease in the triglyceride content of heart that we reported earlier (1). This is evidenced by the findings that the concentrations of plasma lipid of the cardiomyopathic hamster (Table III) were not different from those of the normal ones. Similarly, epinephrine-stimulated lipase activity was the same in the diseased animals as in the normal controls. This suggests that there is no limitation in the supply of free fatty acids that could be made available to the tissues, provided

TABLE VI

Incorporation of sn[U-^{14}C] Glycerol-3-Phosphate[a] (G3P) in Total Lipids[b] by Heart, Muscle, Liver, and Adipose Tissue Homogenates of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamsters

	Age 30 days		Age 185 days	
	RB	BIO	RB	BIO
Heart	6.15 ± 0.28	5.35 ± 0.25	6.28 ± 0.24[c]	5.41 ± 0.26[c]
Muscle	2.23 ± 0.20	1.83 ± 0.17	1.71 ± 0.21[d]	0.94 ± 0.09[d]
Liver	2.63 ± 0.23	2.04 ± 0.14	3.49 ± 0.14	4.21 ± 0.31
Adipose tissue	24.45 ± 2.2[e]	18.58 ± 1.44[e]	10.43 ± 1.06[f]	5.63 ± 1.12[f]

[a]Incorporation expressed as nmoles G3P incorporated/min/mg protein.

[b]All values are $\bar{X}$ ± SEM of at least six observations.

[c-f]Values with similar superscripts are statistically significant ($P<.05$).

TABLE VII

Relative Incorporation of sn[U-^{14}C] Glycerol-3-Phosphate[a] (G3P) in Neutral Glycerides[b] by Heart, Muscle, Liver, and Adipose Tissue Homogenates of Randomly Bred (RB) and Cardiomyopathic BIO 82.62 (BIO) Hamster

	Age 30 days		Age 185 days	
	RB	BIO	RB	BIO
Heart	0.45 ± 0.07	0.43 ± 0.06	0.49 ± 0.04[c]	0.36 ± 0.04[c]
Muscle	0.11 ± 0.012[d]	0.15 ± 0.010[d]	0.063 ± 0.011	0.074 ± 0.018
Liver	0.83 ± 0.05	0.69 ± 0.04	1.05 ± 0.28[e]	1.54 ± 0.15[e]
Adipose tissue	9.00 ± 0.74[f]	6.37 ± 0.82[f]	2.76 ± 0.22[g]	1.60 ± 0.42[g]

[a]Relative incorporation of G3P expressed as nmoles/min/mg protein.

[b]Values are of $\bar{X}$ ± SEM of at least six observations.

[c-g]Values with similar superscripts are statistically significant ($P<.05$).

uptake of free fatty acids from plasma by the tissues is not hampered. Measurement of lipoprotein lipase activity, which is the rate-limiting step in tissue uptake of plasma triglycerides (25,26), shows that no differences exist between the activity of the enzyme of heart and muscle (Table IV) of the cardiomyopathic hamsters and that of normal controls. Our findings are at variance with those reported by Kelley (23). He reported an increase in the activity of the enzyme in the myocardium of myopathic hamsters in severe congestive heart failure and concluded that the elevated LPL was probably an adaptive change induced in the failing heart. The discrepancy between our findings and those of Kelley's could be due to differences in the strains (14.6 vs. 82.62) or in the selection of animals. In our experiments, we randomly picked genetically myopathic hamsters of a certain age without selectively choosing the severely ill ones.

The depression in LPL activity that we observed in adipose tissue (Table IV) of the cardiomyopathic hamsters could limit the supply of fatty acids made available to the tissue and thus partially impair glyceride synthesis. The combined effect of the depression in LPL activity, the depression in de novo fatty acid synthesis that we reported earlier (2), and the depression in the endogenous capacity of the tissue to incorporate G3P (Table VI) apparently is the cause of the noticeable decrease in the size of adipose tissue in the cardiomyopathic hamster (Table II). Both the effects and consequences of such impariment in energy "storage" in the diseased animals warrant further investigation.

The abnormal accumulation of intracellular triglycerides in the muscle of some dystrophic animals is one of the characteristic pathological features of the disease (27). Possible causes of such accumulation have been proposed, including depressed fatty acid oxidation (28) and an elevation in the capacity of dystrophic muscle to synthesize fatty acids from acetate (29). Recently, it was reported (30) that triglyceride synthesis in the muscle of dystrophic mice is not different from normal controls. Results obtained in our experiments with the hamster are at variance with the above findings. We demonstrated that there was a decrease in the total lipid and the triglyceride content of heart but no change in the concentrations of these fractions in the muscle (1). Additionally, de

novo fatty acid synthesis in the liver and adipose tissue of the diseased animals was found to be depressed as a result of the depression in the activities of many of the enzymes that are involved in fatty acid synthesis. Similarly, the results of the present sutdy clearly indicate that de novo glyceride synthesis is depressed in the heart, muscle, and adipose tissue of the cardiomyopathic hamsters (Table VI). These changes, in addition to explaining the possible cause of the decreased lipid and triglyceride content of heart, may suggest that the cardiomyopathic hamster may be suffering from a kind of dystrophy that differs from other dystrophies in relation to alterations in lipid metabolism.

ACKNOWLEDGMENTS

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Kinetics of a Micelle Specific Palmitoyltransferase Isoenzyme of Rabbit Mammary Gland

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ABSTRACT

Palmityl-CoA:monopalmityl-*sn*-glycerol 3-phosphate palmitoyltransferase [EC 2.3.1. -] in rabbit mammary gland microsomes is composed of two isoenzymic species. The α form (LPAT-α) is active with monomeric substrates and inhibited by micelles while the β form (LPAT-β) is active only with micelles. By combining the effects of time, temperature, and Tween 80 which selectively inhibited LPAT-α, the substrate saturation curve for the LPAT-β isoenzyme has been successfully determined. Both theoretical and experimental curves are in good agreement.

INTRODUCTION

The enzyme acyl-CoA:lysophosphatidic acid acyltransferase (LPAT) catalyzes the reaction: acyl-CoA + lysophosphatidic acid $\rightleftharpoons$ phosphatidic acid + coenzyme A. At pH 7.4, both substrates are anionic amphiphiles and a monomer-micelle transition is observed within the substrate concentration range used to define the acceptor saturation (V/S_a curve) of this enzyme (1). Evidence indicates that LPAT in the microsomal fraction of rabbit mammary gland is composed of two isoenzymic species denoted α and β, respectively (1). The α form is active with monomeric or dispersed substrate molecules and inhibited by micelles whereas LPAT-β is active with micelles but not monomers. The dependence of the reaction rate of these two isoenzymes on acceptor concentration corresponds, respectively, to Type III and IV saturation kinetics as defined by Gatt et al. (2). When both species are active in the mammary microsomal fraction with Vmax-β/Vmax-α>0.5 and Km-α<cmc<K-β, the corresponding V/S_a curve becomes biphasic at the critical micelle concentration (cmc) of the substrate (1,3).

The specific activity of the two LPAT isoenzymes in rabbit mammary gland microsomes was monitored as a function of stage in pregnancy and lactation, and LPAT-α activity was observed on all sampling dates (1,3). LPAT-β activity, however, was not evident in mammary tissue from rabbits in early pregnancy (day 7 and 16 of pregnancy) nor in late lactation (35 days post partum) thereby facilitating definition of the V/S_a curve for LPAT-α alone. The corresponding V/S_a curve for LPAT-β, however, was not experimentally obtainable since β activity in native microsomes was at all times accompanied by significant LPAT-α activity. Thus, only a theoretical acceptor saturation curve for LPAT-β could be obtained, and this was secured by the method of graphical resolution (1).

Herein we present details of the methods whereby the V/S_a curve of LPAT-β in rabbit mammary gland microsomes was experimentally determined. LPAT-α activity was selectively inhibited by the combined effects of time, temperature, and nonionic detergency, Tween 80. At limiting LPA concentrations, LPAT-β displayed sigmoid kinetics as predicted in our previous study (1).

EXPERIMENTAL PROCEDURES

Materials

The 4,4'-dithiodipyridine (Aldrithiol-4) was obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI) and Tween 80 from Atlas Chemical Industries, Inc. (Wilmington, DE). The 1-monopalmitoyl-*sn*-glycerol 3-phosphate or lysophosphatidic acid (LPA) was purchased from Serdary Research Laboratories, Inc. (Ontario, Canada). Bovine serum albumin (fraction V powder) was obtained from Sigma Chemical Co., (St. Louis, MO) and Pituitary Luteinizing Hormone was from Armour-Baldwin Laboratories (Omaha, NB).

Animals

Virgin Dutch Belted rabbits, obtained from Rockland Farms, Inc., Gilbertsville, PA, were given pituitary luteinizing hormone (0.5 mg/kg body weight) intravenously via the marginal vein of the ear to stimulate ovulation and immediately bred by artificial insemination. Semen was collected from a colony of Dutch

[1]Systematic names of compounds referred to in the text by their trivial or trade names are as follows: lysophosphatidic acid, monoacyl-*sn*-glycerol 3-phosphate; phosphatidic acid, diacyl-*sn*-glycerol 3-phosphate; Aldrithiol-4, 4,4'-dithiodipyridine; Tween 80, polyoxyethylene sorbitol monooleate; Triton X-100, polyoxyethylene p-t-octyl phenol.

Belted rabbits at the Animal Science Department, and each doe received ca. 0.1 ml undiluted semen. The animals, caged individually in a thermostatically controlled room, were fed Rabbit Chow (Agway, Ithaca, NY) ad libitum throughout pregnancy (31 days) and lactation. The average litter size was 9 (range 6-11).

Preparation of the Microsomal Fraction

The animals were sacrificed by cervical dislocation and immediate decapitation. Mammary gland was excised carefully to avoid contamination with adipose, muscle, or connective tissue. All subsequent procedures were performed on ice. Mammary tissue was washed with 0.25 M sucrose, blotted dry with cheesecloth and weighed. After chopping with a scissors, the tissue was homogenized for 30 sec at high speed in 3 volumes of 0.25 M sucrose on a two-speed Waring Blendor drive unit with stainless steel semimicro jar and blade unit accessories. Tissue that had associated upon homogenization was dispersed by chopping with a scissors and rehomogenizing for a further 30 sec. All associated tissue was then removed from the homogenate, ground in a mortar with glass beads (0.45-0.5 mm diameter, VWR Scientific, Rochester, NY), returned to the homogenate and rehomogenized for 60 sec at high speed. This crude homogenate was centrifuged at 15,000 g (11,250 rpm) for 20 min at 4 C in a Sorvall Superspeed RC2-B centrifuge with fixed angle rotor (Sorval type SS-34, r_{av} 4.25 in). The supernatant was carefully decanted through four layers of cheesecloth and centrifuged at 44,000 g (21,000 rpm) for 1 hr at 4 C in a Beckman L2-65 ultracentrifuge with fixed angle rotor (Beckman type 21, r_{av} 9 cm). The microsomal fraction was collected after careful decantation of the high speed supernatant, suspended in 0.25 M sucrose to 20-30 mg protein/ml using a Ten Broeck tissue homogenizer and immediately analyzed for protein. Aliquots of the suspension were taken for enzyme assay and the remainder stored at -25 C after lyophilization for 48 hr at 50-20 Torr on a Model 10-100 Virtis Unitrap freeze-dryer (VWR Scientific).

Preparation of the Microsomal Suspension

When fresh microsomes were used as the enzyme source, the procedure outlined above was employed. With lyophilized microsomes, appropriate amounts were suspended in 0.25 M sucrose to give a final protein concentration of 20-30 mg/ml, using a Ten Broeck tissue homogenizer and Teflon pestle. Homogenization for ca. 1 min was carried out in an ice bath. Suspended microsomes were then sonicated for 1 min at 0 C in a Model 8845-3 sonicator (Cole-Palmer Ultrasonic Cleaner, Cole-Palmer, Chicago, IL) and stored on ice. Protein determination was made prior to assay since all assays were performed at 0.23 mg microsomal protein/ml.

Protein Determination

Protein was measured using a modification of the Lowry method (4). All measurements were performed in a Bausch and Lomb Spectronic 700 at 750 nm, and BSA (fraction V powder) was used as standard.

Tween 80 had the effect of slightly increasing the A_{750} of protein analyzed by the above method. Thus, when Tween 80 was included in the protein sample, all solutions (standards and sample) were adjusted to 0.035% (w/v) Tween 80. Under these conditions, the standard curve was displaced vertically by ca. 0.04A within the analytical range of 30-150 μg BSA/ml.

Acyltransferase Assay

Unless otherwise stated, the standard reaction mixture contained Tris-HCl buffer, 70 mM, pH 7.4; 4,4′-dithiodipyridine (Aldrithiol-4), 0.3 mM; palmityl-CoA, 18 μM; microsomal protein in 0.25 M sucrose, 0.23 mg/ml; and monopalmityl-*sn*-glycerol 3-phosphate.

The assay method is essentially that of Lands and Hart (5) with the exception that the more sensitive sulfhydryl-binding reagent, 4,4′-dithiodipyridine (Aldrithiol-4), was used. Prior to assay the enzyme was preincubated for 4 min in the reaction medium without substrates. The cells were positioned in a constant temperature cell holder (accessory to the Perkin-Elmer Spectrophotometer Model 356, Hitachi, Ltd., Tokyo, Japan) connected to a circulating water bath (Haake Model FE, VWR Scientific). After the rate of palmityl-CoA hydrolysis was measured (1.5 min), 1-monopalmityl-sn-glycerol 3-phosphate (>99% 1-isomer) was added and the absorbance change at 324 nm recorded continuously for 2-4 min in quartz-suprasil curvettes (1 cm light path), with a 10 mV Hitachi Perkin-Elmer Model 165 recorder attached to a Perkin-Elmer Model 365 spectrophotometer. Operating characteristics of these instruments have been previously described (3).

The specific activity of palmityl-CoA thiolase in rabbit mammary microsomes was consistently low at ca. 2 nmoles/mg protein/min when assayed at 18 μM palmityl-CoA and LPA was shown to depress the activity of this enzyme (3).

According to Grassetti and Murray (6), the

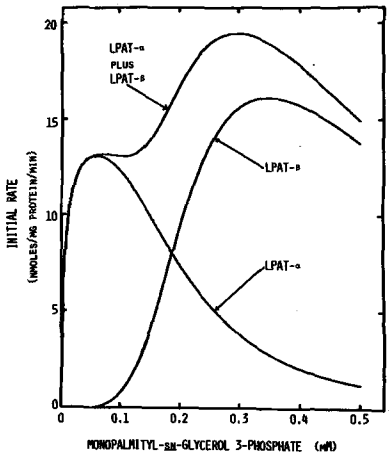

FIG. 1. Acceptor saturation kinetics for rabbit mammary microsomes containing either LPAT-α alone or both LPAT-α and LPAT-β isoenzymes. All curves were generated by computer according to the rate equations presented previously (1) with Vmax-α = 15 nmoles/mg protein/min and Vmax-β = 0 or 20 nmoles/mg protein/min.

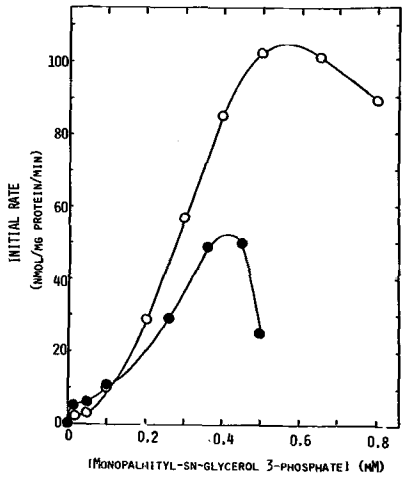

FIG. 2. Acylation of monopalmityl-*sn*-glycerol 3-phosphate by microsomes from lactating rabbit mammary glands without added detergent (●——●) and in the presence of Tween 80, 0.75 mg/ml (o——o). Reaction mixtures as specified under "Experimental Procedures" with monopalmityl-*sn*-glycerol 3-phosphate as indicated. Microsomes were isolated from the mammary gland of an animal 4 days post-partum.

molar absorbtivity (ϵ) of Aldrithiol-4 was 19,800 A/M.cm. However, at an initial A_{324} of 0.5 (i.e., the A_{324} of the reaction mixture without substrates measured against an air blank), the ϵ observed and thus used with this instrument was 17,700 A/M.cm (7).

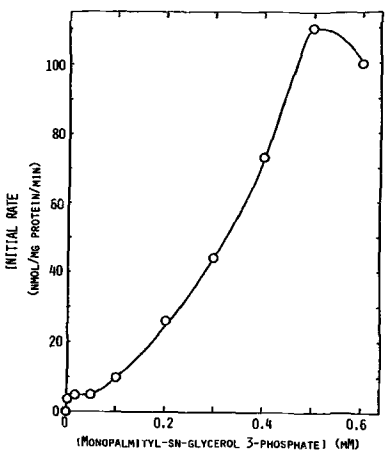

FIG. 3. Acylation of monopalmityl-*sn*-glycerol 3-phosphate by microsomes from lactating rabbit mammary gland at a fixed ratio of Tween 80 and monopalmityl-*sn*-glycerol 3-phosphate (2 mg/μmole). Reaction mixtures and conditions as specified in Figure 2.

Computer Analysis

A Hewlett Packard calculator (Model 9820 A) with cassette memory (Model 9865 A) and X-Y plotter (Model 9862 A) was used to plot the kinetic functions, the parameters of which were determined by trial and error.

RESULTS

The lysophosphatidic acid (LPA) saturation curve for LPAT-β from rabbit mammary gland microsomes was obtained by graphical subtraction of a normalized V/S_a curve for LPAT-α from a biphasic V/S_a curve (Fig. 1). This latter curve represented the composite saturation kinetics of the two LPAT isoenzymic species (1). The theoretical V/S_a curve for LPAT-β was defined by the Hill equation incorporating a term for cooperative, noncompetitive substrate inhibition. Our objective was to obtain experimental data for the V/S_a curve of LPAT-β and the methods employed are described below.

In a preliminary series of experiments, information was obtained which suggested a procedure for the analysis of the acceptor saturation kinetics of LPAT-β. It was observed that when assays were performed at 18 μM palmityl-CoA and 0.4 mM LPA, Tween 80 brought about a 180% increase in LPAT activity. The effect of Tween 80 was, therefore, examined over the entire LPA concentration range at a constant level of added detergent (Fig. 2) and at a fixed ratio of Tween 80 to LPA (Fig. 3). The results show that while LPAT-β activity was doubled, that of LPAT-α was reduced by

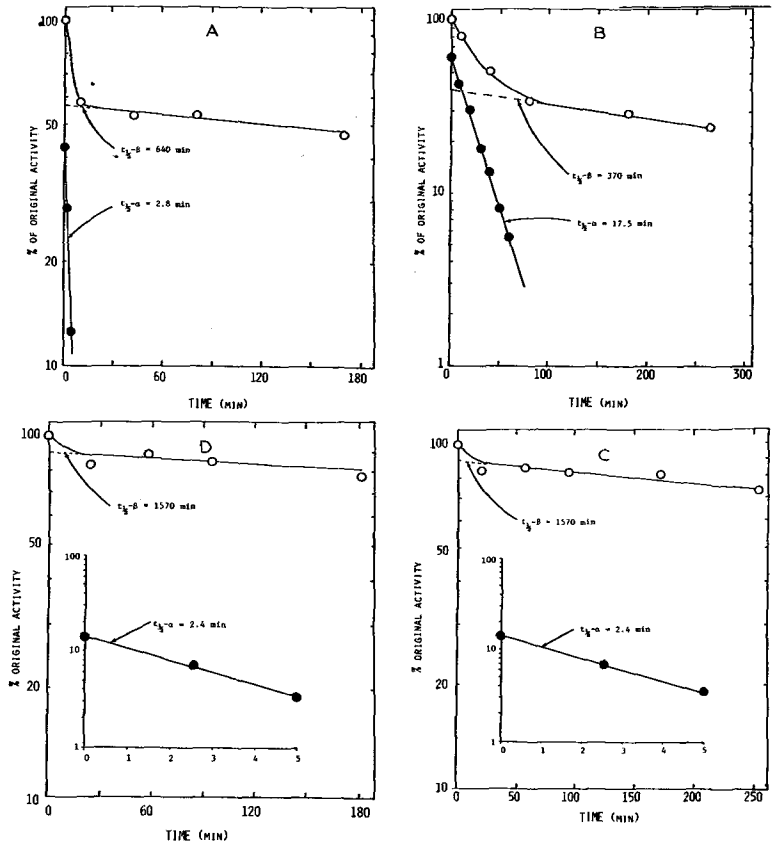

FIG. 4. Thermal stability of palmityl-CoA:monopalmityl-*sn*-glycerol 3-phosphate palmityltransferase with (A and D) and without (B and C) Tween 80 when assayed at 0.05 mM (A and B) and 0.4 mM (C and D) monopalmityl-*sn*-glycerol 3-phosphate. Microsomes (5.4 mg protein/ml) from the mammary gland of a rabbit 9 days post-partum were incubated at 38 C with Tween 80 (17.6 mg/ml) for specified time intervals prior to assay. Assay conditions as specified under "Experimental Procedures" with Tween 80, 0.75 mg/ml and monopalmityl-*sn*-glycerol 3-phosphate as indicated. Each stability curve (o——o) has been graphically resolved into an α (•——•) and a β (- - - -) component and the corresponding half-lives are indicated. Insets in C and D, represent the resolved stability curves of the short-lived α component.

ca. 50% at 0.75 mg/ml Tween 80 (Fig. 2). These data suggested the possibility of selectively inhibiting LPAT-α without adversely affecting LPAT-β activity, thereby facilitating analysis of the LPA saturation kinetics for LPAT-β alone.

Concomitantly, we observed that LPAT-α was less heat stable than LPAT-β. Therefore, heat and Tween 80 treatments of the microsomes were combined to rapidly and selectively inhibit the former isoenzyme. However, in order to determine a suitable incubation period wherein LPAT-α inhibition is maximized and that of LPAT-β minimized, the half-life of the two isoenzymes at 38 C was measured with and without Tween 80 (Fig. 4). Assays were performed at 0.05 and 0.4 mM LPA, i.e., the acceptor concentration corresponding to maximum α and β activities, respectively. At both LPA concentrations, the stability curves were resolved into two components reflecting residual α and β activity at 0.4 and 0.05 mM LPA, respectively (Fig. 4). At 38 C and in the presence of Tween 80, LPAT-α activity decayed with the half-life ($t_{½}$-α) of 2.8 min. The corresponding half-life for LPAT-β ($t_{½}$-β) was 26 hr (Fig. 4).

Considering the relative insensitivity of LPAT-β to the combined heat/detergent treatment, mammary microsomes were incubated for 85 min, i.e., 39$t_{½}$-α, under the above conditions and immediately assayed for transacylase activity as shown in Figure 5. V/S_a curves for untreated microsomes and for microsomes that had been incubated with Tween 80 at 0 C are included for comparison. As expected, LPAT-α activity was essentially eliminated by this treatment (Fig. 5). The observed V/S_a curve repre-

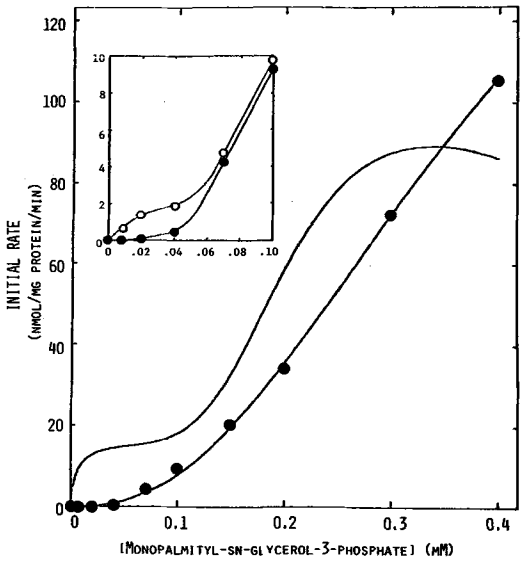

FIG. 5. Acylation of monopalmityl-*sn*-glycerol 3-phosphate by microsomes previously incubated with Tween 80 (17.6 mg/ml) for 85 min at 38 C and 5.4 mg microsomal protein/ml, (●——●, curve A). The corresponding saturation curve for untreated microsomes is also shown (————, curve B). The inset reveals in detail the dependence of reaction rate upon acceptor concentration in the lower acceptor concentration range and includes part of a saturation curve (○——○) obtained with microsomes previously incubated with Tween 80 as above, but at 0 C. Microsomes were isolated from the mammary gland of a rabbit 9 days post-partum. Assay mixtures were as spedified under "Experimental Procedures" with monopalmityl-*sn*-glycerol 3-phosphate as indicated and Tween 80, 0.75 mg/ml. Curves A and B were generated by computer using the rate equation presented in the text.

sents, therefore, the LPA saturation kinetics of the LPAT-β isoenzyme alone (Fig. 5, curve A) and bears a marked resemblance to that graphically resolved and kinetically defined in an earlier study (Fig. 1) (1).

When the treated microsomal suspension was stored on ice, the V/S_a profile presented in Figure 5 (curve A) was stable for up to 5 days and regeneration of LPAT-α activity was not observed.

As indicated by the computer generated V/S_a curve (Fig. 5), a good fit to the experimental data for detergent-treated microsomes was obtained using the Hill equation (8):

$$Vo\beta = \frac{Vmax\text{-}\beta\ S^n}{S^n + K\text{-}\beta}$$

when $Vo\beta$ is the initial reaction velocity; S is the LPA concentration; Vmax-β, the maximum reaction rate possible with a fixed amount of enzyme, = 200 nmoles/mg protein/min; n, the Hill coefficient, = 2.4; and K-β, the Hill constant, = 2.5 mM LPA. For comparative purposes, parameters of the kinetic functions describing the LPA saturation curve for LPAT-β in treated and untreated microsomes are presented in Table I.

TABLE I

Parameters of the Kinetic Functions Describing the Monopalmityl-*sn*-Glycerol 3-Phosphate Saturation Curves of the Palmityl:CoA: Monopalmityl-*sn*-Glycerol 3-Phosphate Palmityltransferase-β Isoenzyme in Tween 80-Treated and Untreated Microsomes[a]

Parameter[b]	Without Tween 80[c]	With Tween 80
Vmax-β	106	200
n	4.5	2.4
K-β	2.5	2.5
m"	3.2	
Ks-β	40	

[a]Treated microsomes had been incubated at 5.4 mg protein/ml with Tween 80, 17.6 mg/ml, for 85 min at 38 C. Assay mixtures were as defined under "Experimental Procedures" without Tween 80 for untreated microsomes and at 0.75 mg Tween 80/ml for treated preparations. Microsomes were isolated from the mammary gland of a rabbit 9 days post-partum.

[b]Vmax-β, nmoles/mg protein/min; K-β and Ks-β (the dissociation constant of the inactive enzyme-substrate complex), mM; m" is the coefficient catering for cooperative substrate inhibition.

[c]Taken from Reference 1.

DISCUSSION

In an earlier series of experiments, we observed that the LPAT-α isoenzyme was active in the microsomes of rabbit mammary gland on all harvesting dates in pregnancy and lactation (3). Thus, the acceptor saturation kinetics of LPAT-β from untreated microsomes could only be defined theoretically by a procedure of graphical resolution (1). Throughout the present study, the nonionic surfactant, Tween 80 was found to attenuate or completely eliminate LPAT-α activity in the microsomal fraction and permitted analysis of the LPA saturation kinetics of LPAT-β exclusively. The experimentally determined V/S_a curve of LPAT-β is similar to the theoretical curve derived previously (1). This, coupled with the differential sensitivities of LPAT-α and β to Tween 80, supports the postulated presence in rabbit mammary microsomes of two LPAT isoenzymic species.

The theoretical LPA saturation curve of LPAT-β in untreated microsomes had been previously described by the Hill equation incorporating a term for cooperative, noncompetitive, substrate inhibition (1). Similarly, with treated microsomes, a good fit to the experimental data points was observed using

the Hill equation. Noteworthy, however, are the differences observed between the kinetic parameters calcualted for treated and untreated microsomes (Table I). For example, a substrate inhibition term was included in the Hill equation describing the V/S_a curve for LPAT-β from untreated microsomes. In the case of treated microsomes, however, an inhibition term was not necessary. This may result from the fact that V/S_a data points at the higher LPA concentrations were not available since the reaction rate was too high for accurate initial velocity measurements to be made. [It was not possible to obtain these data by reducing the the level of microsomal protein in the assay medium because initial rate was not linear with protein concentration (1)]. It may also indicate that in the case of treated microsomes, where assays were performed in the presence of Tween 80, LPA inhibition was simply not operative. The Vmax-β in treated microsomes was almost double that observed for native microsomes. This increase indicates that LPAT-β in native microsomes is held in a partially inhibited form. Results obtained with untreated microsomes, assayed in the presence of Tween 80, demonstrated this same effect (Figs. 2 and 3).

The LPA concentration corresponding to peak LPAT-β activity was increased in the presence of Tween 80 (Figs. 2 and 5). Under similar conditions, the maximum specific activity of LPAT-β was also increased. This treatment of mammary microsomes with Tween 80, however, was not responsible for solubilization of LPAT (9). Thus, the observed differences in the kinetic parameters observed for treated and untreated microsomes do not reflect differences between particulate and solubilized forms of the β-isoenzyme (cf. ref. 10 and 11). It is possible that Tween 80 interacts directly with LPAT-β and, by inducing a conformational change in the protein, alters its kinetic properties. In addition, the manner in which substrates are presented to or products removed from the active site of the enzyme might change in the presence of Tween 80. It is possible also that the available LPA and palmityl-CoA substrates exist with Tween 80 as mixed micelles. Indeed, Lipovac et al. (12) have suggested that the activation of sialidase from *Vibrio cholereae*, observed in the presence of added lecithin, is brought about by a more favorable spacing of sialyl residues in the micelle by nonsialyl lipid. Accordingly, mixed micelles of LPAT substrates and Tween 80 may be more acceptable to the enzyme than pure substrate aggregates. This could, therefore, account for the significant increase in the maximum β activity observed in the presence of detergent.

Kinetic description of a substrate saturation curve by the Hill equation usually implies allosteric activation. However, as noted for the rate equation describing the saturation curve for LPAT-β determined in the absence of Tween 80 (1), it cannot be ascertained, at the present time, whether allosteric activation is real or merely reflects an effect of phase change and a differential reaction rate of isoenzyme-β toward monodisperse and aggregated substrate molecules. Furthermore, as a result of nonspecific binding and micellarization of substrate molecules, the kinetic parameters presented above must be considered complex functions rather than constants. Thus, the significance of the decrease in the Hill coefficient from 4.5 to 2.5 for native and treated microsomes, respectively, cannot be evaluated.

LPAT-α is specific for monomeric substrates and inhibited by the corresponding aggregates or micelles. When Tween 80 was included in the assay medium, the concentration of detergent was always above the cmc, i.e., Tween 80 at 0.75 mg/ml [cmc - 0.013 mg/ml (13)]. Thus, detergent micelles are expected to be present in all such incubation mixtures if nonspecific binding is of little significance. The observed loss of LPAT-α activity in the presence of Tween 80 may, therefore, be accounted for by the fact that this isoenzymic species is sensitive to all micellarized surfactants independent of chemical identity. In contrast, LPAT-β, which is active on micelles, is presumably not adversely affected by the detergent properties of aggregated Tween 80. These observations support our recent proposal (9) that in vivo the level of endogenous surfactants regulates the relative activities of the two isoenzymic species.

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Incorporation and Metabolic Conversion of Erucic Acid in Various Tissues of the Rat in Short Term Experiments

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ABSTRACT

Rats were intravenously injected with a mixture of free (14-^{14}C) erucic acid (22:1) and (9-10^{3}H) oleic acid (18:1). After 2, 4, 8, 16, and 30 min, radioactivity was examined in blood, liver, heart, kidneys, and spleen. Free (^{14}C) 22:1 disappeared from the blood more rapidly than free (^{14}C) 18:1 between 0 and 8 min. Incorporation of label into triglycerides only appeared after 16 min and at 30 min they represented 4% of the injected radioactivity. In this fraction, 63% of ^{14}C radioactivity was present as 18:1 and not as the original 22:1, while almost all ^{3}H radioactivity was recovered as unchanged 18:1. At all times studied, the majority of radioactivity was found in the liver, primarily as triglycerides (60% of radioactivity in total lipids) and as phospholipids (20-30%). ^{14}C was present in nearly the same proportion as ^{3}H (13% of injected radioactivity after only 2 min, 11% at 30 min). ^{14}C radioactivity was contained in 18:1 in higher proportion than 22:1 (45% in triglycerides, 65% in phospholipids). Since labeled triglycerides of blood, rich in (^{14}C) 18:1, mainly originate from the liver triglycerides, it appears that 18:1 is the major form of utilization of 22:1 in the tissues after its conversion in liver. In the other organs tested, radioactivity was found 10-15 times lower than in liver. In the heart, ^{14}C was 3 to 4 times higher than ^{3}H. More than 80% was recovered as 22:1 in triglycerides. In spleen and kidneys, the ^{14}C:^{3}H ratio was particularly high in free fatty acids and monoglycerides. In kidneys, 60% of ^{14}C was present as nervonic acid (24:1) in monoglycerides and 40% in phospholipids, suggesting that the mononervonin formed was used for phospholipid biosynthesis.

INTRODUCTION

Regular ingestion of rapeseed oil, rich in erucic acid, is known to perturb lipid metabolism and cause anatomical lesions in the myocardium (1-4). Relationships between erucic acid in the diet and the existence of certain disorders have been demonstrated (5-8), but the exact role of this acid has not yet been defined (9).

It has been shown that erucic acid is transformed in various organs, primarily the liver, into other monounsaturated fatty acids, particularly oleic acid (10-12) by shortening, and nervonic acid (12-14) by lengthening its chain. However, these transformations were studied only after long experimental times (2 hr minimum) in animals on a high lipid diet (15-10 wt %). Under these conditions, it is not always easy to interpret the results observed.

New studies of the metabolism of erucic acid were thus undertaken during short periods from 2 to 30 min and in rats eating a balanced diet of normal lipid content, by comparing the fate of (14-^{14}C) erucic acid and (9-10^{3}H) oleic acid in various organs after simultaneous injection of these two fatty acids.

MATERIAL AND TECHNIQUES

Experimental Procedures

Male weanling rats of Wistar strain were maintained on a balanced diet (UAR, 91360 Epinay-sur-Orge, France) for 2 mo. Weighing ca. 250 g each, the animals were starved for 12 hr and then injected via a catheter introduced into the jugular vein prior to the experiment, with an equimolar mixture (1.175 μmole) of (14-^{14}C) erucate (24.02 μCi) and (9-10^{3}H) oleate (53.54 μCi) complexed with albumin. The two labeled fatty acids were provided by the Commissariat à l'Energie Atomique (C.E.A., Saclay, France) and the radiopurity was determined to be greater than 98% by gas liquid chromatography (GLC) (15).

Two or three animals were killed at 2, 4, 8, 16 and 30 min after injection, their blood was collected in the presence of heparin by puncturing the abdominal aorta, which was followed by removal of liver, heart, kidneys, and spleen. Each organ was washed with cold 0.9% NaCl solution, dried on filter paper, and weighed.

[1] A preliminary report of this work was presented at the 10th International Congress of Nutrition, Kyoto, Japan, August 1975.

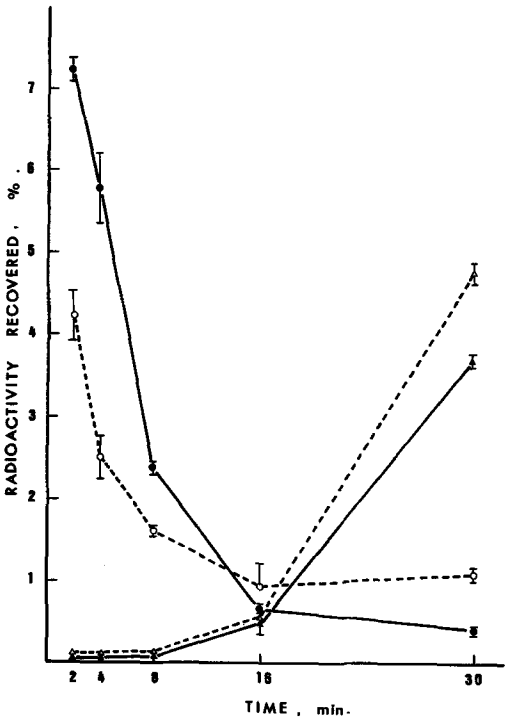

FIG. 1. ^{14}C (————) and ^{3}H (– – –) radioactivity in FFA (○,●) and TG (△,▲) of blood.

Analytical Techniques

The various organs were crushed in a mortar and lipids were extracted, as were those in the blood, using a mixture of dimethoxy-methane-methanol, (4:1, v/v) according to Delsal's technique (16). Aliquots were removed for radioactivity determination.

The total lipids were separated into lipid classes–phospholipids (PL), mono- (MGT), di- (DG) and triglycerides (TG), free fatty acids (FFA) and cholesterol esters (CE) – by thin layer chromatography (Merck Silica Gel G plates, 500 μm thick) using petroleum ether-ethyl ether- acetic acid-methanol (90:20:2:3, v/v) as the developing solvent. The various bands were recovered and the lipids eluted with chloroform-methanol (50:50, v/v) for phospholipids and 90:10 for the other lipids. Then radioactivity of the various lipid classes was measured.

Distribution of radioactivity between the different fatty acids in the PL and TG was determined by preparative gas chromatography of butyl ester derivatives, which were collected at the column outlet (15). A Barber-Colman series 5000 gas chromatograph was used with an argon ionization detector. Glass columns, 1.20 m long and 5 mm in diameter, filled with 25% DEGS on Chromosorb W, 80-100 mesh, were employed at 190 C with 100 ml/min of argon carrier gas.

Radioactivity of the various samples of lipids and fatty acids was measured by liquid scintillation using a Tri-Carb Packard 544 spectrometer with automatic quenching correction. One ml of methanol was added to the PL and MG samples before adding the scintillation liquid (100 ml Permafluor III Packard per 1 liter of toluene) to insure solubilization.

RESULTS

Radioactivity in Blood Lipids (Fig. 1)

^{14}C and ^{3}H radioactivity in the FFA decreased rapidly from 0 to 16 min. ^{3}H radioactivity disappeared more quickly than ^{14}C radioactivity between 0 and 8 min, as shown by the isotopic ^{14}C:^{3}H ratio which was much greater than unity. The isotopic ratio was then observed to be reversed between 16 and 30 min after the injection.

Labeled TG first appeared in the blood after 8 min and in appreciable amounts after 16 min. These TG contained slightly more ^{3}H than ^{14}C.

The other lipids were also analyzed. DG were found to be rapidly labeled and they contained 1% of ^{3}H and 0.6% of ^{14}C radioactivity injected throughout the time period studied. PL were also observed to be rapidly labeled with 0.7% of ^{3}H and ^{14}C at 4 min followed by a decrease in radioactivity. The decrease of ^{14}C was slower than ^{3}H. Thus, radioactivity appears much earlier in blood DG and PL than in TG.

Radioactivity in Total Lipids of the Various Organs (Table I)

Two minutes after injection of the labeled fatty acids, 13% of the ^{14}C radioactivity was recovered in the liver lipids, 10-15 times more than in the other organs. At 30 min, ^{14}C radioactivity of the liver decreased by only 30% as compared to the amount found at 2 min, whereas that of the other organs had fallen by more than 50%. The ^{14}C:^{3}H ratio was about unity in the liver and kidneys, but much higher in the heart and spleen. It tended to decrease with time in all organs studied except the liver.

Radioactivity in Lipid Classes of the Various Organs (Tables II to V)

^{14}C radioactivity in the liver and heart was principally found in TG, especially in the heart, and was distinctly higher than ^{3}H radioactivity in this organ, with a ^{14}C:^{3}H ratio of 3 to 4. PL were labeled more in the liver than in the heart. FFA and MG generally did not contain a high proportion of ^{14}C radioactivity in either organ.

TABLE I

Radioactivity ^{14}C[a] and Isotopic Ratio $^{14}C:^{3}H$[b] in Total Lipids of the Various Organs

Time, min	Liver		Heart		Kidneys		Spleen	
	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$
2	13.1 ± 0.4	1.09 ± 0.08	1.29 ± 0.09	4.07 ± 0.20	1.18 ± 0.15	1.18 ± 0.02	0.89 ± 0.04	5.65 ± 0.50
4	12.8 ± 1.6	0.99 ± 0.04	1.00 ± 0.10	2.88 ± 0.39	0.98 ± 0.03	1.14 ± 0.11	0.65 ± 0.02	4.93 ± 0.19
8	11.7 ± 0.2	1.09 ± 0.18	0.57 ± 0.03	3.35 ± 0.27	0.70 ± 0.04	1.16 ± 0.02	0.50 ± 0.02	5.63 ± 0.43
16	10.2 ± 1.4	1.19 ± 0.06	0.46 ± 0.09	3.04 ± 0.34	0.59 ± 0.03	1.13 ± 0.14	0.26 ± 0.02	2.82 ± 1.74
30	10.8 ± 0.7	1.13 ± 0.05	0.55 ± 0.09	2.89 ± 0.05	0.57 ± 0.02	1.08 ± 0.01	0.37 ± 0.03	2.27 ± 1.59

[a]% of injected radioactivity.

[b]Ratio of the injected fatty acids = 1.00. Values are means ± standard error.

TABLE II

Distribution of ^{14}C Radioactivity between the Different Classes of Lipids of Liver[a] and Isotopic Ratio $^{14}C:^{3}H$[b]

Time, min	PL[c]		MG		DG		FFA		TG + CE[d]	
	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$
2	22.7 ± 2.9	1.04 ± 0.05	6.2 ± 0.9	3.47 ± 0.40	3.4 ± 1.0	0.88 ± 0.08	4.2 ± 1.3	1.59 ± 0.32	63.5 ± 2.5	1.02 ± 0.08
4	21.7 ± 2.1	0.92 ± 0.12	6.1 ± 0.4	3.38 ± 1.79	6.0 ± 1.6	1.45 ± 0.96	8.9 ± 0.5	2.34 ± 0.95	57.3 ± 2.2	0.83 ± 0.00
8	18.7 ± 2.6	0.85 ± 0.10	1.2 ± 0.1	1.02 ± 0.02	8.4 ± 0.2	1.96 ± 0.24	6.2 ± 0.6	2.76 ± 1.30	62.6 ± 0.7	1.07 ± 0.15
16	24.5 ± 1.9	0.95 ± 0.04	0.9 ± 0.0	1.16 ± 0.24	10.7 ± 0.1	3.05 ± 0.20	3.0 ± 0.1	1.65 ± 0.06	60.9 ± 1.4	1.17 ± 0.05
30	32.9 ± 1.9	1.02 ± 0.02	4.3 ± 3.0	1.35 ± 0.00	2.7 ± 2.0	2.54 ± 0.17	3.0 ± 0.0	1.11 ± 0.05	57.7 ± 2.2	1.02 ± 0.12

[a]% of total radioactivity.

[b]Ratio of the injected fatty acids = 1.00. Values are means ± standard error

[c]Abbreviations: PL = phospholipids, MG = monoglycerides, DG = diglycerides, FFA = free fatty acids, TG + CE triglyceride + cholesterol esters.

[d]Radioactivity found in CE in each analysis was very low and to simplify was added to that found in TG.

TABLE III

Distribution of ^{14}C Radioactivity between the Different Classes of Lipids of Heart[a] and Isotopic Ratio $^{14}C:^{3}H$[b]

	PL[c]		MG		DG		FFA		TG + CE[d]	
Time, min	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$
2	5.4 ± 1.0	2.40 ± 0.13	1.7 ± 0.0	2.26 ± 0.42	4.3 ± 0.8	1.84 ± 0.60	6.2 ± 0.3	3.07 ± 0.06	82.4 ± 0.4	4.16 ± 0.26
4	5.4 ± 0.3	1.56 ± 0.41	1.5 ± 0.2	1.97 ± 0.57	3.5 ± 0.8	1.58 ± 0.48	4.6 ± 0.3	1.98 ± 0.08	85.0 ± 1.6	3.39 ± 0.41
8	7.4 ± 2.1	1.76 ± 0.09	1.5 ± 0.4	1.99 ± 0.75	4.8 ± 1.5	1.51 ± 0.18	5.0 ± 0.6	1.89 ± 0.26	81.3 ± 2.6	4.03 ± 0.08
16	6.1 ± 1.0	1.12 ± 0.15	2.3 ± 2.0	1.98 ± 0.47	4.2 ± 1.3	1.54 ± 0.04	4.8 ± 0.1	1.47 ± 0.07	82.6 ± 0.1	3.88 ± 0.83
30	5.2 ± 0.4	0.74 ± 0.04	4.5 ± 1.0	1.97 ± 0.03	2.2 ± 0.1	1.17 ± 0.28	4.2 ± 1.4	1.54 ± 0.05	83.9 ± 3.8	3.53 ± 0.27

[a]% of total radioactivity

[b]Ratio of the injected fatty acids = 1.00. Values are means ± standard error.

[c]Abbreviations: PL = phospholipids, MG = monoglycerides, DG = diglycerides, FFA = free fatty acids, TG + CE = triglycerides + cholesterol esters.

[d]Radioactivity found in CE in each analysis was very low and to simplify was added to that found in TG.

TABLE IV

Distribution of ^{14}C Radioactivity between the Different Classes of Lipids of Kidneys[a] and Isotopic Ratio $^{14}C:^{3}H$[b]

	PL[c]		MG		DG		FFA		TG + CE[d]	
Time, min	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{4}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$	^{14}C	$^{14}C:^{3}H$
2	24.4 ± 9.0	0.70 ± 0.19	16.2 ± 0.4	2.35 ± 0.44	3.7 ± 1.7	1.02 ± 0.27	27.9 ± 7.4	5.55 ± 0.71	27.8 ± 2.6	0.73 ± 0.01
4	19.9 ± 0.9	0.54 ± 0.03	20.9 ± 0.2	2.90 ± 0.36	3.5 ± 0.3	0.98 ± 0.07	24.4 ± 3.9	3.96 ± 0.48	30.5 ± 0.3	0.86 ± 0.11
8	30.5 ± 0.9	0.74 ± 0.15	23.9 ± 0.2	2.60 ± 0.79	3.2 ± 0.1	0.98 ± 0.00	9.8 ± 1.7	3.23 ± 0.49	29.2 ± 4.4	1.04 ± 0.12
16	35.4 ± 0.3	0.73 ± 0.10	22.2 ± 6.0	2.07 ± 0.10	2.8 ± 0.2	0.82 ± 0.17	7.7 ± 0.3	1.59 ± 0.31	31.9 ± 3.2	0.96 ± 0.15
30	28.5 ± 4.0	0.70 ± 0.04	17.0 ± 7.0	4.48 ± 0.15	7.3 ± 0.3	0.69 ± 0.03	6.9 ± 0.7	1.69 ± 0.03	40.3 ± 2.5	0.93 ± 0.04

[a]% of total radioactivity.

[b]Ratio of the injected fatty acid = 1.00. Values are means ± standard error.

[c]Abbreviations: PL = phospholipids, MG = monoglycerides, DG = diglycerides, FFA = free fatty acids, TG + CE = triglyceride + cholesterol esters.

[d]Radioactivity found in CE in each analysis was very low and to simplify was added to that found in TG.

On the contrary, FFA and MG had high ^{14}C radioactivity in kidneys and spleen which was much higher than ^{3}H within the first 8 min. The proportion of ^{14}C radioactivity present in FFA decreased with time in both organs, whereas that of PL and TG increased. There was also an increase with time in MG ^{14}C radioactivity in the spleen, without any corresponding increase in ^{3}H radioactivity. Furthermore, although the proportion of ^{14}C radioactivity in TG of this organ was not high (50% to 20% of the radioactivity recovered in total lipids), it was significantly higher than that of ^{3}H radioactivity, as in the heart, with a ^{14}C:^{3}H ratio greater than 3 in most cases.

Distribution of ^{14}C Radioactivity in the Various Fatty Acids of TG and PL in the Organs (Table VI).

Rapid metabolic transformations of erucic acid (22:1) were observed from the second minute after injection of the labeled acid in all organs except the heart. Oleic acid (18:1) [Identification of the monounsaturated fatty acids formed was made in a previous paper (12).] was the principal fatty acid formed, particularly in the liver where it contained a higher level of ^{14}C radioactivity than the 22:1 in PL and TG. However, a higher proportion of nervonic acid (24:1) was formed in the kidneys from the 22:1. The 24:1 of PL from this organ contained 40% of the ^{14}C radioactivity from the second minute.

In general, other fatty acids such as palmitoleic (16:1) and eicosenoic acid (20:1) were not labeled to any extent.

The same study of radioactivity was carried out on the blood FFA at 6 min and on the blood TG at 30 min. Results showed that 95% of the ^{14}C radioactivity in the FFA was present as unconverted 22:1. In triglycerides, about two-thirds (63%) of the ^{14}C radioactivity was recovered as 18:1, while only 15% remained as unchanged 22:1. The ^{14}C fatty acids of MG from kidneys, which were highly labeled, were also analyzed. The 24:1 was found to be the most highly labeled fatty acid newly formed, containing 60% of the total ^{14}C radioactivity.

DISCUSSION

Our results show that erucic acid injected in a single dose in animals whose diet does not contain this fatty acid is taken up more slowly than oleic acid. However, the difference is not very significant, found to be about the same as that between oleic and palmitic acids as reported by Göransson and Olivecrona (17). Moreover, this slight delay does not seem to

TABLE V

Distribution of ^{14}C Radioactivity between the Different Classes of Lipids of Spleen[a] and Isotopic Ratio ^{14}C:^{3}H[b]

Time, min	PL[c]		MG		DG		FFA		TG + CE	
	^{14}C	^{14}C:^{3}H	^{14}C	^{14}C:^{3}H	^{14}C	^{14}C:^{3}H	^{14}C	^{14}C:^{3}H	^{14}C	^{14}C:^{3}H
2	10.6 ± 0.6	1.45 ± 0.09	7.5 ± 1.5	9.42 ± 3.02	8.2 ± 5.0	1.95 ± 0.77	60.2 ± 3.9	18.87 ± 1.53	13.5 ± 1.8	3.62 ± 0.20
4	13.2 ± 3.2	1.19 ± 0.17	10.9 ± 0.9	7.80 ± 0.56	5.0 ± 0.2	0.77 ± 0.15	58.2 ± 2.6	14.04 ± 2.73	12.8 ± 0.7	2.67 ± 0.59
8	17.4 ± 0.4	2.12 ± 0.08	16.4 ± 0.1	11.92 ± 1.14	5.6 ± 0.8	1.69 ± 0.13	46.4 ± 2.2	14.65 ± 3.36	14.2 ± 0.7	3.81 ± 0.07
16	26.5 ± 1.7	1.75 ± 0.08	24.6 ± 1.8	12.35 ± 3.40	7.6 ± 1.1	1.47 ± 0.38	23.5 ± 2.7	5.54 ± 1.97	17.8 ± 0.4	3.35 ± 0.29
30	36.9 ± 3.0	1.47 ± 0.58	23.8 ± 1.9	15.09 ± 0.00	4.4 ± 0.1	1.22 ± 0.03	15.5 ± 0.5	2.79 ± 0.00	19.4 ± 1.1	3.32 ± 0.06

[a]% of total radioactivity.
[b]Ratio of the injected fatty acids = 1.00. Values are means ± standard error.
[c]Abbreviations: PL = phospholipids, MG = monoglycerides, DG = diglycerides, FFA = free fatty acids, TG + CE = triglycerides + cholesterol esters.
[d]Radioactivity found in CE in each analysis was very low and to simplify was added to that found in TG.

TABLE VI

Distribution of ^{14}C Radioactivity between the Monounsatured Fatty Acids of the Triglycerides and Phospholipids of the Different Organs[a]

Fatty acids	Time, min	Liver		Heart		Kidneys		Spleen	
		TG[b]	PL	TG	PL	TG	PL	TG	PL
16:1	2	2.4	10.3	1.0	3.2	2.1	2.8	2.8	3.4
	4	2.1	6.2	1.2	0.6	2.2	2.7	2.2	4.7
	8	3.1	8.1	0.4	4.6	2.0	3.2	2.4	5.3
	16	3.1	7.7	0.7	5.0	1.8	3.7	2.5	6.6
	30	3.2	8.3	0.9	3.5	3.3	3.9	3.4	7.5
18:1	2	36.3	64.5	5.8	20.8	14.2	10.9	30.6	25.3
	4	57.2	67.4	8.0	26.8	15.0	16.4	24.7	31.4
	8	44.6	55.4	6.9	32.2	17.8	18.2	31.0	37.3
	16	49.6	66.0	10.3	29.4	20.9	22.1	32.7	41.9
	30	46.5	68.5	9.4	32.6	28.8	21.0	31.6	40.5
20:1	2	5.1	5.7	3.4	6.9	5.2	4.8	15.6	9.2
	4	5.4	4.2	2.3	5.3	5.7	7.5	10.2	8.9
	8	5.4	5.9	1.8	5.3	7.6	6.1	9.3	8.7
	16	5.5	4.7	2.3	6.4	8.9	5.1	8.3	8.3
	30	5.1	5.0	1.5	6.3	7.7	4.9	7.5	7.4
22:1	2	41.9	13.4	86.4	59.9	61.4	26.4	40.0	45.9
	4	26.9	10.3	83.8	54.2	59.4	26.4	49.8	41.4
	8	38.8	19.8	84.6	43.2	53.9	24.2	46.1	36.2
	16	37.3	13.7	83.4	49.2	41.0	22.8	44.1	30.4
	30	29.7	11.0	83.7	43.6	39.8	20.1	42.9	26.6
24:1	2	0.7	1.2	1.2	4.5	13.3	43.5	5.6	9.8
	4	1.9	1.2	1.4	4.5	11.4	36.7	6.2	10.0
	8	0.9	4.0	1.3	3.9	11.7	40.0	9.4	8.3
	16	0.8	1.7	1.7	3.8	10.3	39.9	9.3	9.4
	30	1.1	1.5	1.2	4.0	10.4	40.7	12.1	12.0

[a]% of total radioactivity of fatty acids. Erucic acid is only converted into monounsaturated fatty acids; only these acids are reported in the table.

[b]Abbreviations: TG = triglycerides and PL = phospholipids.

persist, since 16 min after injection there is less ^{14}C than ^{3}H radioactivity in blood FFA. The whole organism, therefore, seems to take up erucic acid in much the same way as other common fatty acids, under the conditions used in this work. This conclusion agrees with that of Jaillard et al. (18) concerning non-preferential extraction of erucic acid by the heart after ingestion of rapeseed oil by humans.

On the other hand, this somewhat slower uptake of erucic acid obviously cannot explain the very rapid accumulation of this fatty acid in heart TG after a single injection, an observation which agrees with several authors using different experimental conditions (1). Neither can it explain the high percentage of ^{14}C radioactivity in the kidney and spleen FFA, which is perhaps due to erucic acid stimulation of phagocytal activity of the reticuloendothelial system (19), particularly that of spleen (20). However, it must be admitted that under our experimental conditions, this phagocytic mechanism was not stimulated in liver, since hepatic FFA were not labeled to any extent. The latter result is contrary to that reported by Carroll (21) for observations at 15 and 30 min after injection of ^{14}C erucic acid, and by Lecerf and Bezard (22) for observations at 2 hr after injection of an identical mixture to that used in this work. However, both groups utilized rats given diets containing 15% weight of oil, and it appears to be a correlation between the low fat content of the diet and the absence of marked persistence of ^{14}C radioactivity in liver FFA (unpublished results).

The high ^{14}C radioactivity in kidney and spleen MG, associated with high isotopic ^{14}C:^{3}H ratio, is a new finding concerning erucic acid metabolism. Analysis of radioactivity of MG fatty acids in the kidneys showed that nervonic acid was responsible for 60% of ^{14}C labeling. Since in kidney PL, nervonic acid contained also the major part of ^{14}C, apparently this organ preferentially incorporates nervonic acid, perhaps as mononervonin, into the newly synthesized phospholipids. This acid is already known to be present in sphingolipids (23,24).

Lastly, our experiments showed that transformation of erucic acid into other monounsaturated acids, mainly oleic acid, is very rapid and takes place in all the organs studied except

the heart. This phenomenon has only been observed after long experimental periods (12). Simple calculations show that less ^{14}C erucic acid than ^{3}H oleic acid is found between 0 and 16 min in total lipids of the various organs studied, although ^{14}C radioactivity is higher than ^{3}H radioactivity. The liver obviously plays a central role in these metabolic transformations. Of all the organs studied, the liver contained the highest level of radioactivity throughout the time period studied and the conversion of erucic into oleic acid was always highest in this organ. Furthermore, the liver was essentially responsible for the production of labeled blood TG, which after only 30 min represented ca. 4% of ^{14}C radioactivity injected, about two-thirds of which consisted of ^{14}C oleic acid.

It, therefore, appears that the organism uses erucic acid mainly after metabolic conversion into oleic acid in the liver, which largely compensates for the difference of complete β-oxidation between erucic and oleic acid. However, with regular ingestion of rapeseed oil (4), this process is insufficient to totally eliminate erucic acid from the various tissues. The immediate and high transformation of erucic into oleic acid should perhaps be correlated with the great "specific dynamic action" observed by Tremolieres et al. (26,27) after ingestion of rapeseed oil by humans. The authors' hypothesis of the microsomal origin of this conversion (27), however, has not yet been demonstrated.

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Incorporation of 1-^{14}C-Acetate into C_{26} Fatty Acids of the Marine Sponge *Microciona prolifera*

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ABSTRACT

The incorporation of 1-^{14}C-acetate into the many fatty acids of the marine sponge *Microciona prolifera* was investigated. Probable precursors of 26:2Δ5,9 and 26:3Δ5,9,19 showed high levels of radioactivity, supporting the following pathways for the biosynthesis of C_{26} acids:

$$16{:}0 \rightarrow \rightarrow \rightarrow 26{:}0 \rightarrow 26{:}1\Delta 9 \rightarrow 26{:}2\Delta 5,9$$

$$16{:}1\Delta 9 \rightarrow \rightarrow \rightarrow 26{:}1\Delta 19 \rightarrow 26{:}2\Delta 9,19 \rightarrow 26{:}3\Delta 5,9,19$$

Degradation of the unsaturated C_{26} acids at their double bonds showed that the ^{14}C was concentrated near the carboxyl end of the chain. Hence, chain elongation was the major mechanism for acetate incorporation into these acids.

INTRODUCTION

Our recent investigations (1,2) of fatty acids in the marine sponge *Microciona prolifera* revealed that 48% of its acids have C_{24}-C_{28} chain lengths. Among these are an unusual new family of polyunsaturated fatty acids containing nonmethylene interrupted double bonds. Specific structures identified included: (major components) 26:2Δ5,9; 26:3Δ5,9,19 and (minor components) 24:2Δ5,9; 25:2Δ5,9; 26:3Δ5,9,17; 27:3Δ5,9,19; 27:3Δ5,9,20. Biosynthetic pathways for such acids were proposed (2) based on probable intermediates found in our fatty acid analyses.

We have now completed a series of ^{14}C-acetate incorporation studies on *Microciona* fatty acids. These experimental results provide direct evidence on how 26:2Δ5,9 and 26:3Δ5,9,19 are synthesized by this sponge.

EXPERIMENTAL PROCEDURES

Materials

Microciona prolifera sponge colonies were collected off Barley Point, Navesink, NJ at a depth of ∿1 m during the summer and fall of 1975. The sponges were transported to the laboratory in seawater and used within 2 hr after collection.

Adenosine triphosphate (ATP), reduced nicotinamide adenine dinucleotide (NADH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), coenzyme A (CoA), glutathione, and streptomycin sulfate were purchased from Sigma Chemical Co. (St. Louis, MO). Sodium 1-^{14}C-acetate and 1-^{14}C-oleic acid were obtained from Applied Science Laboratories (State College, PA).

Incubation Conditions

Incubation conditions for sponge experiments were selected to resemble the environment at the collection site as much as possible.

For the intact sponge systems, sponge samples were carefully cleaned and the tips of the fingerlets, 0.2-1.5 g blotted wet weight, were placed in flasks containing 25 ml seawater and 10-25 μCi 1-^{14}C-acetate (55 μCi/mole). The seawater was collected at the sampling site, filtered through 0.22 μm Millipore filters, and buffered at its original pH (c. 8.1) with 0.02 M *tris*-(hydroxymethyl)-aminomethane-HCl.

For the whole cell systems, sponge tips were placed in Ca^{++} and Mg^{++} free seawater, and the cells were disaggregated according to Humphreys (3). The cells were then washed, centrifuged, and the pellet resuspended in a seawater medium identical to that used for the intact sponge incubations.

For the cell-free systems, sponge cell pellets were resuspended in 5 volumes of 0.55 M sucrose containing 20 mM phosphate buffer (pH 7.4) and homogenized using a mechanically driven Teflon-on-glass homogenizer. Cell rupture was monitored with a light microscope. Unbroken cells and debris were removed by centrifugation at 600 x g. The incubation mixture for acetate incorporation contained 6 μmoles $MgCl_2$, 40 μmoles $NaHCO_3$, 20 μmoles ATP, 2 μmoles CoA, 4 μmoles NADH, 4 μmoles NADPH, 2 μmoles glutathione, 40 μmoles phosphate, 1.1 mmoles sucrose, 25 μCi 1-^{14}C-acetate, and 11 mg sponge protein [assayed by microkjeldahl analysis (4)] in a total volume of 2.0 ml.

All incubations were carried out in Dubnoff metabolic shakers at temperatures similar to those of the seawater at the collection site (16-26 C, depending on time of year). Streptomycin was added (50 μg/ml) to prevent

bacterial growth. Incubations were stopped at the desired times by homogenization in 20 volumes of 2:1 chloroform-methanol.

Fatty Acid Analysis

Total lipid was extracted according to the method of Folch et al. (5). Fatty acid methyl esters were prepared by H_2SO_4-catalyzed methanolysis (6) and isolated by thin layer chromatography (TLC). When nonradioactive carriers were necessary, fatty acids isolated previously from *Microciona* (2) were used.

Total methyl esters were fractionated according to degree of unsaturation by TLC on $AgNO_3$-impregnated silicic acid (Ag^+-TLC) and then according to chain length using preparative gas liquid chromatography (GLC) as described previously (2). This procedure separates *Microciona* fatty acids not only according to chain length and number of double bonds but also according to the position of those double bonds in certain fatty acid chains. A detailed discussion of this effect has been given elsewhere (2); however, the 26:1 isomers can be cited here as a typical example. All the 26:1Δ9 present appeared in the monoenes II band, while the 26:1Δ17 and 26:1Δ19 isomers were recovered from the monoenes I band. Hence, the individual chain lengths isolated from each band represented either single or very similar (ω7 + ω9) positional isomers.

Aliquots taken at various stages of the fractionation were evaporated and dissolved in a toluene scintillator (Omniscint, ICN Pharmaceuticals, Inc., Cleveland, OH) for radioassay. All samples were counted in a Beckman LS-230 liquid scintillation counter. Samples with low activity were counted for sufficient time to produce 2σ standard deviation of <5%.

Distribution of ^{14}C Along C_{26} Chains

Unsaturated C_{26} methyl esters resolved by preparative TLC + GLC were converted to dinitrophenylhydrazones by a procedure adapted from Privett and Nickell (7). About 0.5 ml CH_2Cl_2 saturated with ozone at −70 C was added to 0.5 ml CH_2Cl_2 containing 30 to 100 μg of individual radioactive C_{26} methyl esters. Excess ozone was immediately removed by bubbling He gas through the solution. Then 2 mg of triphenylphosphine followed by 1 mg of dinitrophenylhydrazine (DNPH) were added. The reaction mixture was stirred in a vortex mixer, its volume reduced under a stream of He, and then quantitatively transferred to 5 x 20 cm silica gel precoated plates (Quantum Industries, Fairfield, NJ) for TLC. Best results were obtained when the elapsed time between ozonolysis and start of TLC was kept under 5 min. Reaction conditions were optimized using methyl 1-^{14}C-oleate until yields ⩾70% were attained.

The alkyl-hydrazones, alkyl-dihydrazones, and alkyl-ester-hydrazones produced from the reactions of C_{26} unsaturates were then isolated by TLC using solvent systems ranging from 75:25 to 100:0 benzene-diethyl ether. Slight changes in the diethyl ether content of the developing solvent produced large changes in R_f values of the individual hydrazones, permitting clean separation of the desired hydrazones and elimination of the reaction by-products. Bands were recovered and rechromatographed at least three times until a constant specific activity was obtained. Bands were identified by cochromatography with hydrazones of known structure, which were produced by reactions with methyl oleate, methyl 6-octadecenoate, various α-olefinic hydrocarbons, 1,5-hexadiene, 1,7-octadiene, 1,9-decadiene, 1,11-dodecadiene, and methyl esters from *Limnanthes douglasii* (8).

Relative amounts of the hydrazone compounds recovered from TLC were determined by measurement of absorbance at 358 nm (9) in $CHCl_3$. The samples were then radioassayed in the same manner described earlier, except that the minor quenching of ^{14}C measurement by the small amount of hydrazone used was taken into account for all calculations. Division of the measured radioactivity in each fragment by the corresponding absorbance for each hydrazone yielded the relative specific activities from the different parts of a given fatty acid chain. Calculations included corrections for the number of hydrazones per fragment and normalization of values within each chain.

One drawback of this hydrazone derivatization technique was the precipitation of short chain dihydrazones (⩽C_6). These were found to be insoluble in a wide variety of solvents; hence, their relative specific activities could not be determined directly. In such cases, partial hydrogenation of the polyunsaturated fatty acid with hydrazine according to the method of Privett and Nickell (10) was used to produce less saturated molecules from which the desired information could be obtained.

The location of double bonds in unsaturated fatty acids was determined by reductive ozonolysis as described previously (2).

RESULTS AND DISCUSSION

Optimization of Incubation Conditions

Optimization studies of ^{14}C-acetate incorporation into the ultra long chain fatty acids of *Microciona* were carried out using both in vivo

TABLE I

Distribution of ^{14}C Radioactivity Among C_{22}, C_{24}, and C_{26} Fatty Acids Containing 0-3 Double Bonds

System[a]	Percentage of fatty acid radioactivity[b]		
	C_{22}	C_{24}	C_{26}
Intact sponge	14.2	22.2	24.2
Whole cells	17.8	27.3	15.1
Cell-free homogenate	17.6	29.8	6.0

[a]Each system was incubated for 3 hr at 26 C with 25 μCi 1-^{14}C-acetate. A single sponge sample was used to provide aliquots for all three systems. Other conditions and preparation of systems are given in text.

[b]Total fatty acid radioactivity = 100%.

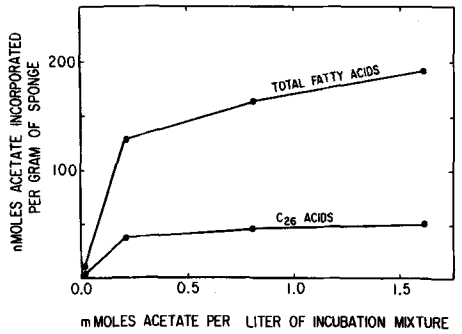

FIG. 1. Effect of acetate concentration in the incubation medium on the incorporation of ^{14}C-acetate into *Microciona prolifera* fatty acids. Intact sponge incubated 10 hr at 20 C with 10-20 μCi 1-^{14}C-acetate.

and in vitro systems. Since almost half of this organism's fatty acids are C_{24}-C_{28}, the distribution of radioactivity according to chain length, specifically among the $\geqslant C_{22}$ acids, was our initial concern.

Table I shows the distribution of ^{14}C in the C_{22}, C_{24}, and C_{26} acids for three different incubation systems acting on aliquots of the same sponge sample. All three systems had the capacity to incorporate relatively high amounts of acetate into the longer chain lengths. Although the distribution of radioactivity for C_{22} and C_{24} acids was relatively equal in all three systems, there was a signifiant drop in the C_{26} radioactivity in the whole-cell and cell-free systems. This lower incorporation of ^{14}C into the C_{26} acids was independent of incubation time and various manipulations of reaction conditions; alterations in the homogenization protocol, use of different buffers, and changes in types and levels of cofactors did not increase it. Since maximum ^{14}C incorporation into the C_{26} acids was most desirable for our C_{26} biosynthetic studies, all further experiments were run using the intact sponge system.

Since nothing was known about the uptake of soluble nutrients such as acetate by *Microciona*, the effect of acetate concentration upon acetate uptake was investigated (Fig. 1). Above 0.2 mM concentration, the rates of acetate incorporation into both the total fatty acids and into the C_{26} acids appeared to be fairly constant, although the relative amount of radioactivity in the C_{26} acids dropped slightly at higher acetate concentrations. Therefore, all subsequent investigations were run with the acetate concentration between 0.2 and 0.3 mM.

Distribution of ^{14}C Among Fatty Acids

To determine the distribution of radioactivity among the major fatty acids after acetate incorporation, 5.3 g of intact sponge was incubated for 4 hr at 16 C with 25 μCi of ^{14}C-acetate. This larger sample size allowed subsequent separations to proceed without the addition of nonradioactive carriers. Fatty acid methyl esters prepared from this sample were then separated using Ag^+-TLC followed by preparative GLC. Gas chromatograms of the individual TLC bands confirmed band identifications and showed mass distributions of fatty acids quite similar to those reported earlier (2). Radioactivity measurements for the various fractions separated are reported in Table II. Results for all C_{26} acids were further confirmed by a second analysis in which the sequence of Ag^+-TLC and GLC separations was reversed.

An unexpected finding in this current fractionation of *Microciona* fatty acids was that the C_{26} material from the dienes II band could be further separated by Ag^+-TLC into two bands. The very minor (~0.7 wt %), faster moving, second band was isolated and identified as 26:2Δ9,19 based on GLC analysis of the aldehydic products from ozonolysis and TLC identification of their DNPH hydrazone derivatives. This 26:2Δ9,19 isomer had not been found previously (2). A possible explana-

TABLE II

Distribution of ^{14}C Radioactivity Among *Microciona prolifera* Fatty Acids[a]

	% of Fatty acid radioactivity[b]						
		Monoenes		Dienes			
Chain length	Saturates	I	II	I	II	Trienes	Total
16	3.1	0.1	2.8	tr	tr	tr	6.0
18	4.7	0.1	12.9	tr	0.1	tr	17.8
20	3.2	0.7	1.7	tr	0.3	0.4	6.3
22	4.3	4.8	0.3	0.1	0.5	0.8	10.8
24	2.9	14.7	0.2	0.8	0.6	1.4	20.6
26	0.5	11.1	6.0	1.6	4.2[d]	1.8[e]	25.2
Others[c]	6.6	1.8	1.3	0.6	0.6	1.0	11.9

[a]Intact sponge incubated 4 hr at 16 C with 25 μCi of 1-^{14}C-acetate.

[b]Total fatty acid radioactivity = 100%. Tetraenes + pentaenes + hexaenes = 1.4%.

[c]C_{14},C_{28}, branched chain, and odd carbon number fatty acids.

[d]Further separated by Ag^+-TLC into 3.3% 26:2Δ9,19 and 0.9% 26:2Δ5,9.

[e]Further separated by Ag^+-TLC into 1.6% 26:3Δ5,9,19 and 0.2% 26:3ω3.

tion for its appearance here might be the seasonal changes in 26:3Δ5,9,19 content of *Microciona* tissues (11) and the probable role of 26:2Δ9,19 as a precursor of 26:3Δ5,9,19 (2). The sample analyzed earlier (2) was collected in early summer when 26:3Δ5,9,19 levels are lower, whereas the sample analyzed in this study was harvested in late fall when 26:3Δ5,9,19 levels are higher.

The overall distribution of ^{14}C radioactivity among *Microciona* fatty acids according to chain length and unsaturation indicates that the enzyme system for chain elongation of fatty acids is especially active in this organism. Over 80% of the total ^{14}C was incorporated into C_{18}-C_{26} acyl chains that are normally considered to be products of chain elongation; and over 45% of the total ^{14}C was found in the C_{24} and C_{26} chains.

We recently proposed (2) biosynthetic pathways for 26:2Δ5,9 and 26:3Δ5,9,19 in *Microciona* based upon known patterns of fatty acid biosynthesis (12-15) and upon the presence of probable precursor acids in this organism. To evaluate these proposals, the total ^{14}C found in each of the precursor and product acids and their relative specific activities (% of total fatty acid ^{14}C/% of total fatty acid mass) have been tabulated in Tables III and IV. Almost all of the postulated precursors and their unsaturated C_{26} products contained substantial levels of total radioactivity. Altogether, the fatty acids listed in Tables III and IV accounted for over 79% of the total fatty acid ^{14}C found, indicating that they are undergoing active biosynthesis. Both these findings support the proposed pathways.

The specific activities of the acids in Tables III and IV show that the precursor acids (16:0 → 26:1Δ9 and 16:1Δ9 → 26:2Δ9,19) were all labeled to a similar degree. However, the specific activities of product acids 26:2Δ5,9 and 26:3Δ5,9,19 were an order of magnitude

TABLE III

Radioactivity Found in Fatty Acids of Proposed Pathway for 26:2Δ5,9 Biosynthesis in *Microciona prolifera*[a]

Fatty acid	% of Total fatty acid ^{14}C	Specific activity
16:0	3.1	0.8
18:0	4.7	2.2
20:0	3.2	4.0
22:0	4.3	1.4
24:0	2.9	1.5
26:0	0.5	5.0
26:1Δ9	6.0	10.0
26:2Δ5,9	0.9	0.05

[a]Intact sponge incubated for 4 hr at 16 C with 25 μCi of 1-^{14}C-acetate.

TABLE IV

Radioactivity Found in Fatty Acids of Proposed Pathway for 26:3Δ5,9,19 Biosynthesis in *Microciona prolifera*[a]

Fatty acid	% of Total fatty acid ^{14}C	Specific activity
16:1Δ9	2.8	4.0
18:1Δ11	12.9	3.4
20:1Δ13	2.4	4.0
22:1Δ15	4.8	9.6
24:1Δ17	14.7	3.9
26:1Δ19	11.1	2.5
26:2Δ9,19	3.3	4.7
26:3Δ5,9,19	1.6	0.1

[a]Intact sponge incubated for 4 hr at 16 C with 25 μCi of 1-^{14}C-acetate.

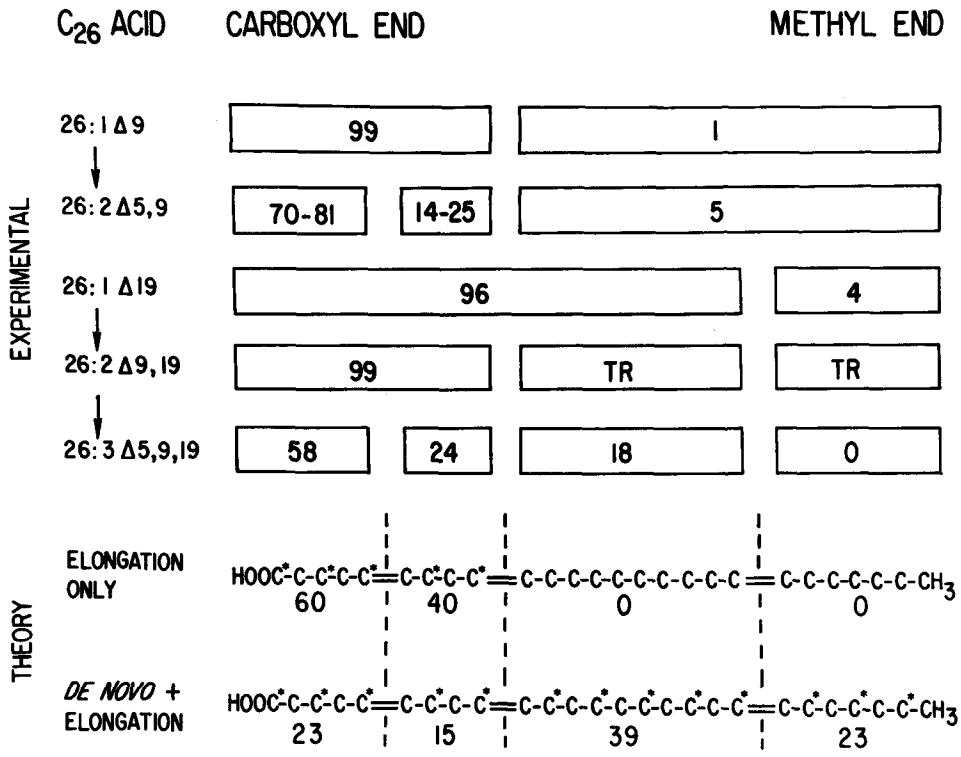

FIG. 2. Distribution of ^{14}C radioactivity along the chains of the unsaturated C_{26} fatty acids of *Microciona prolifera*. Numbers indicate percent of activity found in each fragment after cleavage of the double bonds. Total ^{14}C activity in each chain = 100%. Values for 26:2Δ5,9 represent limits of specific activity based on calculations described in text.

less. This is not merely the result of dilution by the large pools of 26:2Δ5,9 and 26:3Δ5,9,19 present, since the total ^{14}C found in these two acids was considerably lower than found in most of the precursor acids. This suggests that Δ5 desaturation, the last step in both proposed pathways, occurs more slowly than the previous elongation and Δ9 desaturation steps.

It should be noted that the monoene fractions assayed for the 26:3Δ5,9,19 pathway actually represented a mixture of the ω7 and ω9 isomers with ω7≫ω9. Hence, the radioactivity data in Table IV represents the sum of both 26:3Δ5,9,19 and 26:3Δ5,9,17 biosynthetic activity. We have previously pointed out the probable presence of such a parallel pathway for ω9 acids in *Microciona* (2).

The ^{14}C distribution reported in Table II also provides information on the biosynthesis of two other groups of fatty acids in *Microciona*. The combined tetra-, penta-, and hexaenoic polyunsaturates had a low specific activity (0.06), implying that 4-6 double bond acids originated in the diet and are incorporated directly into the sponge lipids without further chain elongation. On the other hand, the C_{24} and C_{26} dienes and trienes produced by elongation of linoleic and linolenic acids were apparently undergoing more active biosynthesis, since the 24:2ω6, 26:2ω6, and 26:3ω3 fractions showed specific activities of 2.7, 1.0, and 0.5, respectively.

Distribution of ^{14}C Along C_{26} Chains

To determine the extent to which chain elongation and de novo biosynthesis pathways contribute to C_{26} fatty acid biosynthesis in *Microciona*, each unsaturated C_{26} acid was cleaved at its double bonds; and the relative specific activities of the resulting fragments were measured (Fig. 2). Since the ozone-triphenylphosphene-dinitrophenylhydrazine treatment used did not permit direct isolation of the C_4 fragment between Δ5 and Δ9 in 26:2Δ5,9 and 26:3Δ5,9,19 (see above), its activity was determined by difference. For 26:3Δ5,9,19 this involved partial hydrogenation with hydrazine (26:3Δ5,9,19 → 26:1Δ5 + 26:1Δ9 + etc.) prior to ozonolysis, so that the relative specific activities of both the C_5 and C_9 alkyl-ester-hydrazones could be measured and their difference (the C_4 fragment) calculated.

With 26:2Δ5,9 there was an additional problem with trace amounts of high activity 26:2Δ9,19 in the low activity 26:2Δ5,9 sample, probably due to tailing of the faster-moving 26:2Δ9,19 band when the two compounds were separated by Ag^+ TLC. Hence, determinations of radioactivity in the C_9 ester-hydrazone from partially reduced 26:2Δ5,9 produced inaccurate results. As an alternative, the radioactivity of the Δ5-Δ9 segment was estimated from the C_{17} and C_{21} alkyl-hydrazones derived from the methyl end of partially reduced 26:2Δ5,9. These C_{17} and C_{21} hydrazones were partially separated by TLC, and the relative specific activities of the two fractions were measured. Using the equation $C_4 = C_{21} - C_{17}$, minimum and maximum C_4 specific activities were calculated for both TLC fractions assuming a broad compositional range of $0\% \leqslant C_{17} \geqslant 90\%$ and $10\% \leqslant C_{21} \geqslant 100\%$ (mole %). Combining the higher minimum with the lower maximum gives the narrow range of relative specific activity for the C_4 fragment reported in Figure 2. Similar maximum/minimum calculations on unfractionated C_{21}/C_{17} alkyl-hydrazone mixtures from two different hydrazine hydrogenations of 26:2Δ5,9 (one to high 26:1 content, the other to low 26:1 content) yielded almost identical results for the specific activity range of the C_4 fragment.

The distribution of ^{14}C in the 26:1Δ9 and 26:2Δ5,9 chains was quite similar. 95-99% of the radioactivity was located in the first nine carbon atoms at the carboxyl end of the chain, indicating that the chain elongation pathway was the main route for ^{14}C-acetate incorporation into these acids. However, the ^{14}C in the 26:2Δ5,9 was more concentrated in the first five carbons at the carboxyl end than one would expect from a simple 16:0 → 26:0 elongation process. Apparently elongation from precursors longer than C_{16} occurred, indicating that the chain elongation system of *Microciona* will accept preformed C_{20}-C_{22} substrate acids. The above findings are in agreement with our proposed 16:0 → 26:2Δ5,9 biosynthetic pathway, and they strongly support a precursor/product relationship for 26:1Δ9 and 26:2Δ5,9.

The distribution of radioactivity in 26:1Δ19, 26:2Δ9,19, and 26:3Δ5,9,19 also pointed to chain elongation as the main process for 1-^{14}C-acetate incorporation into these acids. 96% of the ^{14}C in 26:1Δ9 was found in the 19 carbon segment at the carboxyl end of the chain. The 26:2Δ9,19 and 26:3Δ5,9,19 contained 99% and 82%, respectively, of their ^{14}C activity in the first nine carbons of the chain. As with 26:2Δ5,9, the ^{14}C in the first five carbons of 26:3Δ5,9,19 was considerably greater than in the Δ5-Δ9 fragment (58% vs. 24%), indicating chain elongation of $\geqslant C_{20}$ substrates.

However, the presence of 18% radioactivity in the Δ9-Δ19 segment of the 26:3Δ5,9,19 chain was unexpected and puzzling. This could not be explained by full de novo biosynthesis of a C_{16} precursor, since the seven carbon unit at the methyl end of the chain contained no radioactivity at all. Chain elongation of a C_{14} or shorter precursor (14:0 → 16:0 → 16:1Δ9 → → → 26:1Δ19 → → 26:3Δ5,9,19) is a distinct possibility. Since none of the other C_{26} unsaturates were found to be similarly labeled, perhaps multiple systems for fatty acid elongation, such as those found in mouse brain (16), may occur in *Microciona*. From an overall point of view, however, the data of Figure 2 tend to support our proposed pathway for the biosynthesis of 26:3Δ5,9,19 in *Microciona*. The ^{14}C labeling patterns in the 26:1Δ19, the 26:2Δ9,19, and in most (82%) of the 26:3Δ5,9,19 are as expected. Nevertheless, the presence of a minor alternative route for 26:3Δ5,9,19 production cannot be ruled out.

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Levels of Cyclic 3'-5'-Adenosine Monophosphate (cAMP) in Maintenance Cultures of Rat Hepatocytes in Response to Insulin and Glucagon

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ABSTRACT

Under conditions of short-term stimulation of lipogenesis by insulin in maintenance cultures of hepatocytes from starved rats, basal levels of cyclic 3'-5'-adenosine monophosphate (cAMP) are invariant. Glucagon signals increased levels of cAMP and severely diminishes lipogenesis. Insulin partially counteracts both glucagon effects.

INTRODUCTION

Insulin has been implicated in the long-term, in vivo control of lipogenesis in liver (See references 1 and 2 for reviews). Coordinate induction of a set of lipogenic enzymes in liver, e.g., acetyl CoA carboxylase (E.C. 6.4.1.2), ATP-citrate lyase (E.C.4.1.3.8), and malic enzyme (E.C. 1.1.1.40), begins 3 hr after feeding starved rats or after the provision of insulin to diabetic rats (1-3). Glucagon and cyclic 3'-5'-adenosine monophosphate (cAMP) block this induction (2). Short-term control events precede the onset of induction (4-8) including effector control (e.g., stimulation of acetyl CoA carboxylase activity by citrate) and the dephosphorylation of certain interconvertible enzymes, e.g., acetyl CoA carboxylase (9), fatty acid synthetase (10), and pyruvate dehydrogenase (11).

Recently we have demonstrated that physiological concentrations of insulin accelerate the incorporation of [1-^{14}C]acetate and [^{3}H]H_2O into fatty acids by hepatocytes of starved rats maintained as monolayers in vitro in Hams medium supplemented with fetal calf serum (12,13). Low levels of glucagon blocked the insulin effect.

Since these in vitro endocrine-initiated changes in lipogenesis were brought about in less than 2 hr, it seemed probable that only short-term control systems were operative. In the present study, we have examined fluctuations in cAMP concentration in cultured hepatocytes under conditions in which lipogenesis is responsive to insulin and glucagon.

METHODS AND MATERIALS

Male Wistar rats, 250 to 300 g in weight, were starved for 24 hr. Hepatocytes were isolated and cultured as described previously (12). In incorporation studies, 4 ml of cell suspension were added to 60 mm dishes, whereas, for cAMP studies, 10 ml of cell suspension were added to 100 mm dishes. The usual experimental design was to plate hepatocytes with Hams medium supplemented with 15% fetal calf serum (14); remove the medium by aspiration after 4 hr; and add back an identical volume of fresh medium. No hormones were added to the medium during the plating period. The incubation period was started by adding hormones and other reagents, dissolved in the same culture medium, in 50 μl quantities. After appropriate time intervals, incubations for incorporation studies were terminated as described earlier (12). Synthesis of fatty acids was monitored by measuring the rate of [1-^{14}C] acetate of [^{3}H]H_2O incorporation (12) essentially as described by Alberts et al. (15). In experiments designed to measure cAMP levels, medium was also removed by aspiration and the dish quickly rinsed once with 10 ml of ice-cold 0.15 M KCl. Subsequently, cells were killed with 0.5 ml of ice-cold 0.5 N trichloroacetic acid, scraped off with a rubber policeman and the whole mixture transferred to a test tube. After neutralization of the deproteinized supernatant, cAMP was assayed by the protein binding assay of Gilman (16).

Collagenase, Type II, was obtained from Worthington (Freehold, NJ); [1-^{14}C]acetate and [^{3}H]H_2O from Amersham (Searle Co. Arlington Heights, IL) components of the culture medium from Flow (Rockville, MD); all other reagents from Baker (Phillipsburg, NJ). Insulin and glucagon were gifts from Dr. Walter Shaw (Eli Lilly Labs., Indianapolis, IN).

RESULTS AND DISCUSSION

Insulin at physiological concentrations (1,16,18) signaled an increase in the rate of incorporation of [1-^{14}C]acetate and [^{3}H]H_2O into fatty acids by in vitro cultures of hepato-

TABLE I

Influence of Insulin, Glucagon, and Cyclic 3'-5'-Adenosine Monophosphate (cAMP) on Fatty Acid Synthesis in Hepatocyte Maintenance Cultures[a]

Experiment	Additions	Fatty acids	
		[1-^{14}C]acetate	[^{3}H]H_2O
I	None	1.33 ± 0.09	3.31 ± 0.07
	Insulin (1.7 nM)	2.48 ± 0.16	5.05 ± 0.63
	Glucagon (0.5 nM)	0.35 ± 0.02	1.71 ± 0.21
	Glucagon (1 nM)	0.24 ± 0.02	1.48 ± 0.42
	Insulin (1.7 nM) + Glucagon (0.5 nM)	0.95 ± 0.15	2.71 ± 0.42
	Insulin (1.7 nM) + Glucagon (1 nM)	0.61 ± 0.04	1.90 ± 0.23
II	None	1.85 ± 0.11	4.03 ± 0.34
	cAMP (1 mM)	0.40 ± 0.05	1.20 ± 0.28

[a]After the 4 hr plating period, medium was removed by aspiration and 4 ml fresh medium added together with hormones or cAMP where indicated and [1-^{14}C]acetate (2.5 μCi, final specific radioactivity 0.96 mCi/mmole, including 0.24 mM endogenous acetate in the fetal calf serum) or [^{3}H]H_2O (10 mCi, final specific radioactivity 45 μCi/mmole). After an additional hour incubations were stopped. Fatty acid synthesis is expressed as nmoles of acetate incorporated/h/mg protein. Values for [^{3}H]H_2O incorporation were converted to acetate equivalents by multiplying with 1.15 (28). Each point represents the average value ± standard deviation of three incubated plates.

cytes isolated from starved rats (Table I). Since the induction of synthesis of lipogenic enzymes in vivo (e.g., malic enzyme, citrate lyase, and the fatty acid synthetase complex) does not commence until 3 hr after insulin injection (1,2,19), it was reasonable to conclude that the changes observed in these hepatocyte cultures involved short-term control mechanisms. This was supported by the fact that cycloheximide (50 μM) did not inhibit the insulin enhancement of acetate incorporation during the first hour of incubation of cultured hepatocytes. In this first hour, glucagon depressed the baseline rate of lipogenesis as well as the elevated rate obtained with added insulin (Table I). Cyclic AMP added to hepatocyte cultures depressed fatty acid synthesis both from [1-^{14}C] acetate and [^{3}H]H_2O (Table I) as previously observed in liver cell suspensions (8). As to lipogenic rates, it should be stressed that in our hands hepatocytes in suspension and in maintenance culture are equally sensitive to both hormones provided incubation media are supplemented with fetal calf serum (12). We prefer the use of maintenance cultures simply because they offer a convenient system in terms of changing the medium and recovering the cells.

Since both acetyl CoA carboxylase (9) and fatty acid synthetase (10) have been described as being interconvertible between phosphorylated (inactive) and nonphosphorylated (active) states, it was of interest to determine the concentration of cAMP in cultured hepatocytes under conditions in which insulin and glucagon affect the rate of lipogenesis.

It was observed that insulin over a wide range of concentrations had no appreciable effect on the basal level of cAMP after 5 min of incubation (Fig. 1, curve A). Insulin at 8.5 x 10^{-8} M did not perturb the basal cAMP level over the entire 1 hr incubation span (Fig. 2). These results essentially ruled out the idea that the primary short-term stimulation of lipogenesis by insulin was mediated by changes in the concentration of cAMP under these in vitro conditions.

As anticipated, at concentrations of 10^{-10} M and higher, glucagon caused a significant elevation of cAMP levels (Fig. 1, curve B). The time course of change in cAMP concentrations in response to two levels of glucagon is presented in Figure 2. It was of interest that both the peak elevation of cAMP at 5 min and the sustained elevation at 60 min were glucagon dose-dependent. These results indicate that in our experimental system the sensitivity of liver cells to glucagon is equal to that of perfused liver (22,23) and of hepatocytes in suspension (24). Depression of the rate of lipogenesis by the concentrations of glucagon employed (Table I) was correlated with elevated levels of cAMP.

In the presence of insulin, glucagon did not diminish the rate of lipogenesis to the same degree as seen with glucagon alone (Table I). Indeed, the rate of lipogenesis with insulin plus glucagon together was over twice that with glucagon alone at 0.4 and 1.0 nM. In the presence of glucagon, higher levels of insulin did not restore the depressed rate of lipogenesis to normal values (12,13).

The level of cAMP in the presence of 0.5 nM glucagon was depressed ca. 12% by the simultaneous addition of 1.7 nM insulin (Fig. 3). At higher concentrations of insulin, the glucagon-

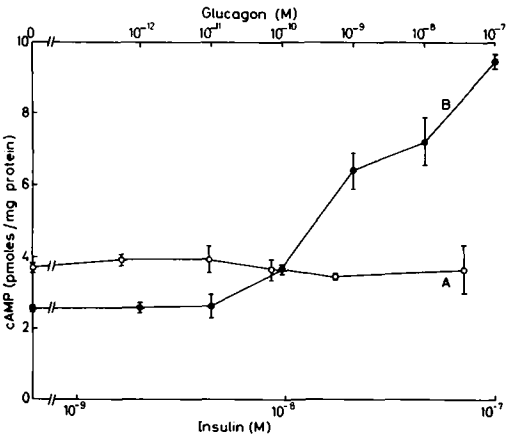

FIG. 1. Effect of insulin and glucagon on cellular cyclic 3'-5'-adenosine monophosphate (cAMP) levels. After the 4 hr plating period medium was removed by aspiration and 10 ml fresh medium added together with hormones. In experiment A insulin was added (0-0); in experiment B glucagon was added (●-●). After incubation for 5 min reactions were stopped. Each point is the mean of at least three separate plates. The vertical bars represent standard deviations.

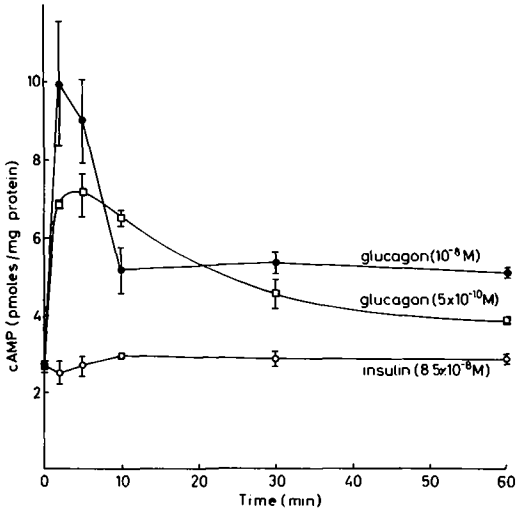

FIG. 2. Fime course of the effect of insulin and glucagon on cellular cyclic 3'-5'-adenosine monophosphate (cAMP) levels. Cells were treated as described in Figure 1. After the indicated incubation times cells were killed (see Methods).

supported levels of cAMP were reduced up to 40%. This limited effect of insulin on cAMP levels was previously observed by Pilkis et al. (24) with hepatocytes (from fed rats) that were suspended in a Krebs-bicarbonate medium. In the present study hepatocytes (from 24-hr starved rats) were cultured for 4 hr in a complete medium (supplemented with amino acids, vitamins, and fetal calf serum) before the addition of insulin and/or glucagon. (See footnote to Table I.)

These results indicate that in cultures of hepatocytes from starved rats insulin can stimulate lipogenesis without affecting cAMP levels. On the other hand, glucagon severly depresses lipogenesis under circumstances that are associated with elevated cAMP levels. Since insulin has a limited capacity to reverse these two effects of glucagon, in this in vitro system it is clear that elevated cAMP, per se, precludes the full expression of the insulin signal.

Dampening of lipogenesis as a consequence of elevated cAMP levels is most easily interpreted in the context of phosphorylation of key enzymes (20). Phosphorylation (inactivation) of acetyl CoA carboxylase (9) and fatty acid synthetase (10) would explain both diminished [1-^{14}C]acetate and [^{3}H]H_2O flux to fatty acids. Furthermore, the impairment of key interconvertible enzymes preceding citrate formation, e.g., pyruvate kinase (21) and pyruvate dehydrogenase (11), would diminish the availability of cytosolic citrate for acetyl CoA carboxylase activation (8,25,26).

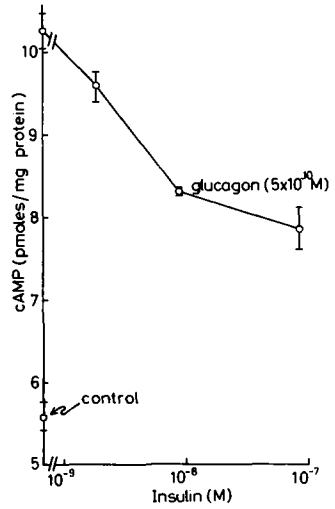

FIG. 3. Effect of insulin on glucagon-elevated cyclic 3'-5'-adenosine monophosphate (cAMP) levels. Cells were treated as described in Figure 1. Both hormones were added at 4 hr. After a 5 min incubation period reactions were stopped (see Methods). Control point: no hormones added.

Insulin stimulation of lipogenesis cannot be explained uniquely in terms of its capacity to lower cAMP levels in the present experimental system. Insulin could conceivably facilitate net dephosphorylation (activation) of several of the interconvertible enzymes cited above by mechanisms not involving cAMP-sensitive protein kinases (27).

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Effect of Dietary Di-2-Ethylhexyl Phthalate on Oxidation of ^{14}C-palmitoyl CoA by Mitochondria from Mammalian Heart and Liver

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ABSTRACT

Oxidation of [1-^{14}C] palmitoyl CoA by heart and liver mitochondria from rats fed dietary di-2-ethylhexyl phthalate (DEHP) was investigated in vitro. Oxidation of ^{14}C-palmitoyl CoA to $^{14}CO_2$ increased two- to threefold in hepatic mitochondria from rats fed 0.1% DEHP for 2 to 3 days; this increase appeared to be a maximum response since similar data were obtained using hepatic mitochondria from rats receiving 0.5% or 1.0% DEHP in the diet. The response of hepatic mitochondria to DEHP was found to continue throughout the duration of 35-day trials in which 1.0% DEHP was fed. In contrast to hepatic mitochondria, the oxidation of ^{14}C-palmitoyl CoA by heart mitochondria decreased ca. 40% upon addition of 0.1% or 0.5% DEHP to the diet; this effect of DEHP on heart mitochondria was not sustained beyond ca. 8 days of DEHP feeding. Limited studies were also performed in rabbits and pigs. Oxidation of ^{14}C-palmitoyl CoA was increased ca. twofold in hepatic mitochondria from rabbits fed 1% dietary DEHP for 12 days and in hepatic mitochondria from pigs that received 5 doses of DEHP (0.8g/kg) at 12-hr intervals; the oxidation ^{14}C-palmitoyl CoA by heart mitochondria from these same animals was unchanged in the rabbit but increased an average of 37% in the pig. DEHP feeding to rats was associated with increased yields of hepatic mitochondrial protein; standardized preparations of heart mitochondria were not similarly affected.

INTRODUCTION

Di-2-ethylhexyl phthalate (DEHP) has a wide variety of industrial uses (1,2) but is probably best known in its role as a plasticizer in the production of polyvinylchloride plastics (2). As a consequence of its widespread usage, DEPH has become an environmental contaminant (3). Unfortunately, little information is available on the biological significance of DEHP in the environment. Recent studies indicate that DEHP can modify lipid metabolism in rats and mice (4-8). Although man (9,10), rats (11,12), and other animals (13) can metabolize DEHP to urinary excretion products, the rate of clearance of DEHP from tissues is variable (10-12,14); 24 hr after a single oral (9,12) or intravenous (12) administration of ^{14}C-DEHP to rats, highest tissue levels are found in liver, adipose, and intestine. DEHP has also been found to accumulate in bovine heart mitochrondria (15) and heart, adipose, and intestinal tissue of humans exposed to plastic medical devices during medical treatment (10,14). The affinity of heart and liver for DEHP prompted the present study in which we examined the effects of dietary DEHP on the oxidation of ^{14}C-palmitoyl CoA to $^{14}CO_2$ by isolated mitochondria from heart and liver of the rat, rabbit, and pig.

EXPERIMENTAL PROCEDURES

Animals and Diets

All animals were male and were individually housed with free access to food and water. Rats (Sprague-Dawley, 250-275 g) were fed either a stock diet (Purina Chow) or the stock diet containing 0.1%, 0.5%, or 1.0% di-2-ethylhexyl phthalate (DEHP)(5); rabbits (New Zealand, 2.5-3 kg) received a stock diet (Purina Chow, pellets) or the stock diet containing 1.0% DEHP. Yorkshire pigs (30-32 kg) were maintained on a stock diet and given 5 doses of DEHP (0.8g/kg) spaced at 12-hr intervals. Each dose of DEHP was mixed into 300 g of the stock diet which was placed into an empty feed trough; the animals consumed the feed within ca. 5 min.

Rats were killed with a blow to the head whereas rabbits and pigs were killed by an overdose of pentobarbital administered intravenously. Liver and heart tissue was removed from the animals immediately after death and minced with scissors in a chilled solution of 0.25 M sucrose, pH 7.4. 20% homogenates (w/v) of liver and heart tissue were prepared in 0.25 M sucrose using a loose-fitting glass Potter-Elvejhem

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TABLE I

Effect of Dietary Di-2-Ethylhexyl Phthalate (DEHP) on the Oxidation of ^{14}C-Palmitoyl CoA by Rat Liver Mitochondria, In Vitro[a]

Duration of DEHP feeding (days)	Dietary level of DEHP (%)					
	0.1		0.5		1.0	
	$^{14}CO_2$ production (Relative %)[b]					
0 (control)	100		100		100	
2	284	(1)[c]	---		---	
3	191±2	(3)	---		---	
4	299	(1)	548	(2)	---	
8	---		272±16	(3)	---	
10	---		199±21	(3)	---	
16	---		329	(2)	227±15	(3)
35	---				278	(2)

[a]Mitochondria were isolated from livers of male Sprague-Dawley rats (250-275 g) that were fed, ad libitum, either a stock diet (Purina Chow) or the stock diet containing DEHP. Mitochondrial oxidation of 1-^{14}C-palmitoyl CoA (sp. act. 56 mCi/mM) to $^{14}CO_2$ was measured in incubations performed at 37 C for 3 hr; the incubation conditions and collection of $^{14}CO_2$ are described in detail in Methods.

[b]$^{14}CO_2$ production (dpm/mg protein/10^5 dpm of ^{14}C-palmitoyl CoA added) is expressed relative to control values obtained in parallel incubations.

[c]Values represent single observations, means or means ± SEM for the number of animals, n, shown in parentheses.

homogenizer. Mitochondria were isolated from the homogenates by centrifugation at 8500 xg after an initial centrifugation of 850 xg (16). The mitochondria were washed once and resuspended in one-third the initial volume of sucrose. The resulting suspensions of heart and liver mitochondria contained ca. 2 mg/ml and 10 mg/ml of protein, respectively. A soluble cofactor solution was prepared by boiling the 8500 xg supernatant from which the mitochondria were sedimented (17).

Incubations

Incubations were performed for 3 hr at 37 C, with shaking, in stoppered 25 ml Erlenmeyer flasks with centre-wells. The incubation mixtures consisted of 1.0 ml of mitochondrial suspension, 3.0 ml of boiled supernatant, and 3.0 ml of 0.25 M tris (hydroxy-methyl) aminomethane-HCl, pH 8.5, containing 3 mg NAD (β-nicotinamide adenine dinucleotide, Sigma Chemical Co., St. Louis, MO, cat. no. N7004) and 1 x 10^5 dpm of ^{14}C-palmitoyl CoA (palmitoyl-1-^{14}C, 56.0 mCi/mmole, New England Nuclear Corp., Boston, MA); penicillin and streptomycin were added at levels of 50 units and 50 μg/ml, respectively.

Analyses

After incubation, 0.5 ml of Protosol (Cat. no. NEF-935, New England Nuclear Corp., Boston, MA) was injected through the stoppers into the centre-wells as a $^{14}CO_2$ trapping agent; $^{14}CO_2$ was displaced from the incubation mixtures by injecting 1.0 ml 6N H_2SO_4 into the flasks. After shaking the flasks for an additional 2 hr, the centre-well contents were dissolved in liquid scintillation fluid (18) and assayed for radioactivity (18).

In each experiment, ^{14}C-palmitoyl CoA oxidation by mitochondria from control and DEHP-fed animals was calculated as dpm $^{14}CO_2$ produced/mg mitochondrial protein (19); in some experiments (Table I, Fig. 1), these data were expressed relative to control values taken as 100% in order to facilitate comparison of data between experiments. $^{14}CO_2$ activity recovered in incubations containing boiled mitochondrial suspensions was deducted from experimental values and amounted to ca. 0.07% of the ^{14}C activity added to the incubations.

RESULTS AND DISCUSSION

The oxidation of 1-^{14}C-palmitoyl CoA to $^{14}CO_2$ was examined in vitro in mitochondria isolated from hearts and livers of rats, rabbits, and pigs fed dietary DEHP.

Liver Mitochondria

The addition of DEHP to a stock diet of rats at levels ranging from 0.1% to 1.0% resulted in an enhancement of ^{14}C-palmitoyl CoA oxidation by hepatic mitochondria. In rats fed 0.1% DEHP, oxidation of ^{14}C-palmitoyl CoA increased two- to threefold over control values (Table I); this increase in oxidation developed

TABLE II

Effect of Dietary Di-2-Ethylhexyl Phthalate (DEHP) on the Oxidation of ^{14}C-Palmitoyl CoA by Isolated Mitochondria from Heart and Liver of Rabbit and Pig[a]

Species	Diet	$^{14}CO_2$ Production (dpm/mg protein)[b] Liver	Heart
Rabbit	Control	33±11(3)[c]	2347±291 (4)
	DEHP	78±25 (4)	2243±302 (4)
Pig	Control	81 (66-96)[d]	1193 (1036-1350)
	DEHP	138(98-178)	1641 (1570-1711)

[a]Male NewZealand rabbits (2.5-3 kg) received, ad libitum, a diet containing 1.0% DEHP for 12 days and male Yorkshire pigs (30-32 kg) received 5 doses of DEHP (0.8 g/kg body wt) mixed with 300 g of hog chow as described under Methods.

[b]Oxidation of 1-^{14}C-palmitoyl CoA (sp. act. 56 mCi/mM) to $^{14}CO_2$ was examined by incubating isolated heart and liver mitochondria with 100,000 dpm of ^{14}C-palmitoyl CoA for 3 hr at 37 C as described under Methods.

[c]Values are means ± SEM of the number of rabbits given in parentheses.

[d]Values are an average of data derived from two pigs; the range of values is given in parentheses.

within 2 to 3 days after initiation of DEHP feeding and was maintained at ca. the same level in studies of 16 and 35 days with diets containing 0.5% and 1.0% DEHP, respectively (Table I). These results suggest that hepatic mitochondria are stimulated maximally with low levels (0.1%) of DEHP in the diet. Based on an average feed consumption of 15 g/day, rats receiving 0.1% DEHP in the diet would have a daily intake of DEHP equivalent to 1/650th of their acute oral LD_{50} which is ca. 30g/ kg (9). Stimulation of hepatic mitochondrial fatty acid oxidation by DEHP feeding is not limited to the rat. In rabbits fed 1.0% DEHP for 12 days, oxidation of ^{14}C-palmitoyl CoA, in vitro, by hepatic mitochondria was increased about twofold (Table II). An increase in fatty acid oxidation was also observed in hepatic mitochondria from two pigs given five oral doses of DEHP at 12-hr intervals (Table II); this increase ranged from a marginal elevation to a twofold difference when compared to data from two control pigs.

The mechanism of DEHP stimulation of fatty acid oxidation in hepatic mitochondria is not evident from the studies presented here but may be related to changes in hepatic carnitine palmitoyltransferase activity since the rate of long-chain acyl CoA oxidation in mitochondria is determined by the rate of acylcarnitine formation (20,21). Of particular interest is a recent report by Reddy et al. (8) who found carnitine acetyltransferase activities to be elevated in liver of rats and mice fed 4.0% DEHP in the diet; no studies of fatty acid oxidation were performed, however.

Modifications of hepatic lipid metabolism by DEHP are not limited to fatty acid oxidation. We found that DEHP feeding to rats for 10 or 18 days significantly reduced the incorporation (5), and altered the distribution (5,22) of ^{14}C-acetate into hepatic lipids in vitro and inhibited hepatic sterol biosynthesis from ^{14}C-acetate and ^{3}H-mevalonate in vitro (7).

An inhibitory effect of DEHP feeding on hepatic sterol biosynthesis has been confirmed in vivo in DEHP-fed rats injected intraperitoneally with acetate-1-^{14}C (F.P. Bell, unpublished observations). These effects of DEHP on hepatic lipogenesis probably account for the reduction of plasma cholesterol and triglycerides observed in rats and mice fed DEHP (8).

Although the effects of DEHP on lipogenesis in vitro are consistent with in vivo observations as noted above, the data reported here on hepatic mitochondrial fatty acid oxidation (Tables I and II) do not so readily explain other known effects of DEHP in liver. For example, Stein et al. (4) found that lipid accumulation occurred in livers of rats fed a 4% fat diet containing 0.1% DEHP as compared to 4% fat-fed controls; an increase in fatty acid oxidation with DEHP feeding would be expected to decrease, rather than increase, hepatic lipid accumulation. It would be possible, however, for increases in lipid oxidation to be masked in such diets if DEHP feeding increased intestinal absorption of fats or promoted a redistribution of tissue lipids to the liver since either of these changes could result in delivery of an increased lipid load to the liver.

In addition to the biochemical changes observed in hepatic mitochondria with DEHP feeding, we consistently observed increases in the yield of mitochondrial protein from livers of DEHP-fed rats (Table III); the increases

TABLE III

Effect of Di-2-Ethylhexyl Phthalate (DEHP) on Yields of Mitochondrial Protein from Rat Liver

Diet	Mitochondrial protein[a] mg/ml
Control	8.65±0.32 (17)[b]
0.1% DEHP	11.33±0.64 (4)
0.5% DEHP	12.94±0.56 (10)
1.0% DEHP	15.59±0.88 (3)

[a]Mitochondria were isolated by a standardized method (see Methods) from livers of male Sprague-Dawley rats (250-275 g) fed either the stock diet (Purina Chow, control) or the stock diet containing DEHP. Protein measurements were made on the resulting mitochondrial suspensions.

[b]Values are means ± SEM of the number of animals shown in parentheses. Duration of DEHP feeding ranged from 2 to 17 days (0.1%, 2 to 4 days; 0.5%, 4 to 17 days; 1.0%, 16 days).

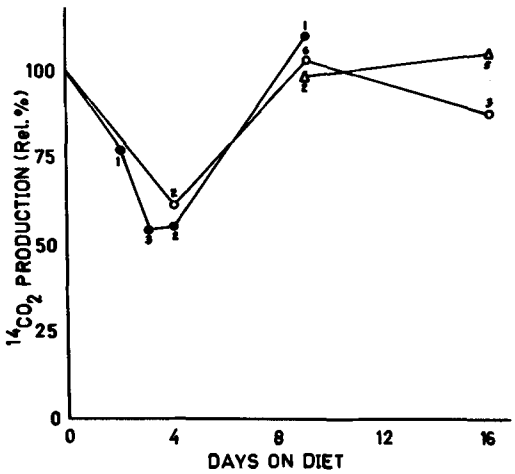

FIG. 1. Effect of dietary di-2-ethylhexyl phthalate (DEHP) on oxidation of ^{14}C-palmitoyl CoA by rat heart mitochondria, in vitro. Mitochondria were isolated from hearts of Sprague-Dawley rats (250-275 g) that were fed ad libitum, a stock diet (control, Purina Chow) or the stock diet containing DEHP at levels of 0.1% (-●-●-), 0.5% (-○-○-) or 1.0% (-△-△-) for periods up to 16 days. Mitochondrial oxidation of 1-^{14}C-palmitoyl CoA (sp. act. 56 mCi/mM) to $^{14}CO_2$ was measured in incubations performed for 3 hr at 37 C as described in Methods. $^{14}CO_2$ production (dpm/mg protein/10^5dpm of ^{14}C-palmitoyl CoA added) is expressed relative to control values obtained in parallel incubations. Numbers below points on the graphs represent the number of DEHP-fed animals studied.

ranged from 30% to 80% above control values (Table III) and were probably the combined effect of DEHP dosage and duration of DEHP feeding. We did not observe any tendency for hepatic mitochondrial protein yields to be elevated in the small number of rabbits and pigs fed DEHP in these studies.

Although we have not examined hepatic ultrastructure in these rats, higher protein yields in mitochondrial preparations may reflect mitochondrial proliferation which is associated with DEHP-induced hepatomegaly (5,8) and proliferation of smooth endoplasmic reticulum (8) observed in rodents. Differences in yield of hepatic mitochondrial protein in these studies emphasize the importance of basing all data on a mg protein basis.

Heart Mitochondria

The oxidation of ^{14}C-palmitoyl CoA was also studied, in vitro, in heart mitochondria isolated from DEHP-fed animals. In rats fed either 0.1% or 0.5% DEHP in a stock diet, ^{14}C-palmitoyl CoA oxidation declined rapidly to about 45-60% of control values in 3 to 4 days (Fig. 1); this inhibition of fatty acid oxidation by heart mitochondria was transient in nature and did not persist beyond 8 or 9 days of DEHP feeding. Normal or near normal values for ^{14}C-palmitoyl CoA oxidation were observed from 9 to 16 days of feeding DEHP at levels of 0.1, 0.5, and 1.0% in the diet (Fig. 1). These data indicate that mitochondria from rat heart and liver respond differently to DEHP feeding; whereas a sustained increase in fatty acid oxidation is observed with hepatic mitochondria (Table I), a transient inhibition of fatty acid oxidation occurs in heart mitochondria (Fig. 1). This transient inhibition may be attributable to decreased activity of carnitine palmitoyltransferase or carnitine, which together control the overall rate of fatty acid oxidation by rat heart mitochondria (20). In preliminary studies we have found the addition of DL-carnitine at a final concentration of 2.6 mmolar to stimulate ^{14}C-palmitoyl CoA oxidation ca. 10-25% in incubations of heart mitochondria derived from rats fed DEHP for 5-6 days.

Oxidation of ^{14}C-palmitoyl CoA was also examined, in vitro, in heart mitochondria isolated from rabbits fed 1.0% DEHP for 12 days (Table II). As in rats fed 1.0% DEHP for 9-16 days (Fig. 1), oxidation of fatty acid was found to be similar to control values (Table II). Since studies were not performed with rabbits fed DEHP for less than 12 days, the presence of a transient inhibition of fatty acid oxidation, as seen in rats, was not observed.

Studies in the pig, although limited to 4 animals, suggest a species difference in the response of heart mitochondria to DEHP. In isolated heart mitochondria from 2 pigs given 5 oral doses of DEHP (0.8g/kg body wt) at 12-hr intervals, ^{14}C-palmitoyl CoA oxidation was

elevated 30-40% above the mean control value (Table II).

In contrast to our observations with hepatic mitochondria (Table III), yields of heart mitochondrial proteins were similar in both control and DEHP-fed rats, rabbits, and pigs.

Although one cannot readily extrapolate in vitro derived data to the in vivo condition, the possibility that DEHP can modify oxidation of fatty acid by heart mitochondria becomes a point of concern since fatty acids are an important energy source for myocardium (23). In this regard, it is of interest that DEHP accumulation has been reported in heart tissue from various animals (15) and man (14). In addition, toxicity of DEHP to cultured heart cells (24) and perfused hearts (25) has been reported. Rat heart displays multiple toxicity effects when perfused with buffer containing DEHP at levels of ca. 0.1 μg/ml (25); the effects include 40-50% decreases in heart rate, glycogen, creatine phosphate, and ATP, all of which could be related to a failure to oxidize sufficient fatty acid as an energy source. DEHP has also been reported to cause death of cultured chick embryo heart cells when present in the culture medium at a level of 4 μg/ml (24).

The data presented here re-emphasize the fact that phthalate esters, commonly used as plasticizers, are biologically active and capable of modifying lipid metabolism in mammalian systems (4-8,26). The association of phthalate esters with toxicity effects in components of the blood and cardiovascular systems (14,25-30) is of particular importance since patients receiving medical treatment with products stored in PVC (polyvinylchloride) plastic containers or PVC plastic delivery devices can be exposed to (10,14,31-34), and accumulate phthalates such as DEHP in blood, lung, and myocardium (10,14).

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Type and Amount of Dietary Fat Affect Relative Concentration of Cholesterol in Blood and Other Tissues of Calves

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ABSTRACT

Sixteen male Holstein calves consumed by nipple a reconstituted milk containing nonfat dry milk and either soybean oil or tallow for 24 weeks. Calves fed milk in this manner (with no dry feed) are functionally nonruminants because the milk bypasses the ruminoreticulum. The fat in these diets contributed about 30% of total dietary calories. The calves consuming soybean oil milk had significantly greater amounts of cholesterol in blood plasma and adipose tissue than did the calves consuming tallow milk.

INTRODUCTION

In man, blood cholesterol level seems to be positively correlated with severity of atherosclerosis. Because an increase in the ratio of polyunsaturated fat (PUF) to saturated fat (SF) in the diet generally is associated with a decrease in blood cholesterol, there has been much emphasis on the seeming merits of increasing the proportion of PUF in the diet of man.

One means of increasing the PUF/SF ratio in man's diet is the use of foods in which this ratio is high. Most plant fats fulfill this requirement, but most animal fats, particularly those from ruminants, do not. The PUF content of ruminant fat can be increased substantially, however, by diets containing unsaturated fat, which is protected from hydrogenation in the ruminoreticulum (1-5).

PUF consumption may affect blood cholesterol levels through several mechanisms. Grundy (6) reported that the consumption of polyunsaturated fats, as compared with saturated fats, caused an increased fecal excretion of endogenous neutral and acidic steroids in most patients and may cause blood cholesterol to be redistributed from blood to other tissue pools (7). It has been noted in rats (8-10), rabbits (11,12), and dairy calves (13) that substitution of PUF for the more saturated fats caused a decrease in blood cholesterol and a concurrent increase in tissue cholesterol levels. In our laboratory, rats fed nonfat dry milk supplemented with a PUF, soybean oil, had a greater blood cholesterol as well as a greater tissue cholesterol level than rats fed a nonfat dry milk supplemented with a more SF, beef tallow (14). Redistribution of blood cholesterol to other tissues may be one mode of action of PUF in lowering blood cholesterol.

A previous study (13) has shown that cholesterol content of edible tissues in growing calves nipple-fed soybean oil (SBO) filled milk was significantly greater than that of similar tissues of calves fed tallow (T) milk. Blood cholesterol was higher, however, in calves fed T. The fat source provided about 50% of total dietary calories.

The present study was designed to determine the effects of diets in which 30% of the calories were from PUF (SBO) and SF (T) on blood and tissue cholesterol concentrations in dairy calves fed reconstituted milks by nipple-pail.

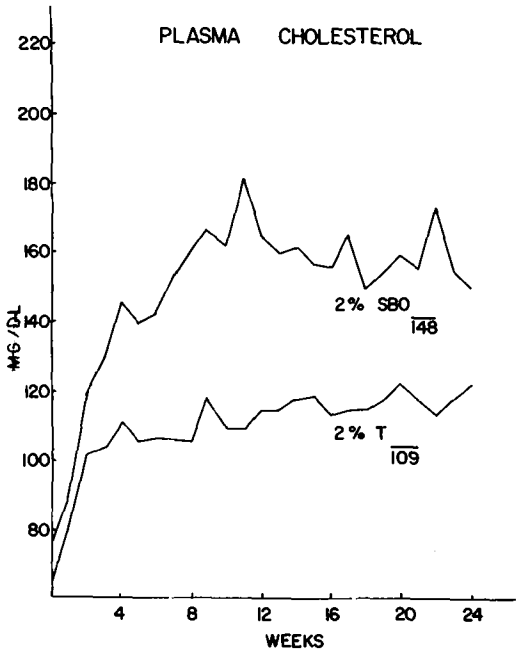

FIG. 1. Mean blood plasma cholesterol (eight calves per group; SBO = soybean oil, T = tallow). SE from analysis of variance = 10.4.

MATERIALS AND METHODS

Over a period of 10 mo, 16 male Holstein

TABLE I

Analysis of Variance of Blood Components and Average Daily Weight Gain for 16 Calves for a Period of 24 Weeks

Source of variation	Cholesterol df	Cholesterol F	Ca df	Ca F	Mg F	Average daily gain df	Average daily gain F
Diet	1	27.3[a]	1	.30	2.61	1	12.20[a]
Calf/diet	14	(5291)[d]	14	(5.74)	(.48)	14	(.12)
Week	24	7.1	6	1.03	12.97[a]	23	2.52[b]
Diet X week	24	.9	6	.95	2.48[c]	23	.59
Residual	330	(686)	84	(4.08)	(.14)	320	(.20)

[a]$P < .005$

[b]$P < .01$

[c]$P < .05$

[d]Figures in parentheses are mean squares for error used to test significance.

TABLE II

Liver Weight and Cholesterol and Body Weight

Group[a]	Liver wet weight	Total liver cholesterol	Body weight (kg) Initial	Body weight (kg) Average daily gain[b]
Soybean oil	2,504 g ± 201	11.6 g ± .87	43.2 ± 1.8	.51 ± .03
Tallow	2,453 g ± 132	12.2 g ± .61	43.8 ± 1.1	.63 ± .03

[a]Eight calves per group.

[b]± SE.

calves ranging from 4 to 10 days of age were allotted alternately, as received, to two comparable groups and fed either SBO- ("Edsoy", Staley Mfg. Co., Decatur, IL) or T- (ISU Meat Lab) reconstituted milk by nipple-pail for 24 wk. Milk fed in this manner bypasses the ruminoreticulum; functionally, the calves are nonruminants (15-17). Milk was fed in two equal feedings at a daily rate of 100 g per kg body weight. Each fat was homogenized (Manton-Gaulin homogenizer, Gaulin Corp., Everett, MA) into the reconstituted milk so that the resulting milk contained 2% fat and 12% nonfat dry milk solids (Formula 202, Low Heat Grade A Non-fat Dry Milk, Assoc. Milk Prod., Inc., San Antonio, TX). Diets were supplemented with water-dispersible vitamins A, D, and E (Each calf received daily per kg body weight: 200 IU vitamin A, Rovimix A-300W; 20 IU vitamin D_3, Rovimix AD_3 300/300W; and 10 IU vitamin E, Rovimix E-100W, Hoffmann-La Roche Inc., Nutley, NJ); chlortetracycline (.4 mg chlortetracycline daily per kg body weight, Diamond Shamrock Chemical Co., Newark, NJ); trace mineral block (given ad libitum, composition in %: NaCl, 98.5; $CoCO_3$, .011; Zn, .005; MnO, .25; FeO, .25; CaO, .33; $CaIO_4$, .007; and MgO, .06) and magnesium oxide (MgO, U.S.P., powder, 17 mg MgO daily per kg body weight, J.T. Baker Chemical Co., Phillipsburg, NJ) Supplementation of B vitamins was not deemed necessary (18).

Jugular blood samples were taken, and body weights were recorded weekly. Analyses for plasma cholesterol, calcium, and magnesium contents were conducted as described previously (13). Calves were slaughtered at the end of the treatment period; the liver, aorta, muscle (biceps femoris), brain, intermuscular fat (adjacent to the biceps femoris), perianal fat, and omental fat were analyzed (13) for cholesterol concentration. Aortae were bisected sagitally; half was stained with Sudan IV, and the other half was analyzed for cholesterol concentration.

Portions of liver biopsies from four calves of each group were incubated with ^{14}C-acetate to determine comparative rates of ^{14}C-incorporation into free fatty acids and cholesterol (19). The other portions of these liver biopsies were immediately frozen by dry ice and later analyzed (Dr. Jon Story, the Wistar Institute, Philadelphia, PA) for 7 α-hydroxylase activity.

The data were analyzed as a split-plot design (20) in which the pooled variation among animals treated alike was used to test the sig-

TABLE III

Means and Standard Errors of Tissue Cholesterol Expressed as Percentage of Dry Matter

Group[a]	Liver	Omental fat	Intermuscular fat	Perianal fat	Muscle	Brain	Aorta	Coronary
Soybean oil	1.82 ± .11	.40 ± .03[b]	.36 ± .02[c]	.42 ± .07[d]	.36 ± .02[e]	10.05 ± .25	.44 ± .02	.54 ± .03[e]
Tallow	1.81 ± .08	.25 ± .02[b]	.25 ± .03[c]	.27 ± .02[d]	.32 ± .01[e]	10.27 ± .22	.42 ± .01	.48 ± .02[e]

[a]Eight calves per group.
[b]Significantly different at $P < .005$.
[c]Significantly different at $P < .05$.
[d]$P = .08$
[e]$P = .09$

nificance of dietary fat. The effects of repeated measurements on the same animals across the 24 wk of the experiment and the diet by week interaction were tested for significance by the residual error.

RESULTS

The T and SBO milk contained a calculated 6 mg and 2 mg cholesterol, respectively, per 100 g milk. Approximately 30% of dietary calories were derived from the respective fat in contrast to about 50% in a previous study (13). There were about 60 kcal per 100 g milk in this study compared with about 64 kcal per 100 g milk in the previous study in which 3.5% fat milks were fed (13).

The least-squares mean plasma cholesterol (148 ml/dl) of the 2% SBO-fed calves was significantly greater ($P<.005$) than cholesterol (109 mg/dl) of 2% T-fed calves (Fig. 1).

Although the calves' diets were supplemented with magnesium, blood magnesium decreased significantly ($P<.005$) with time on diets (Table I). There was no significant effect of dietary fat on blood magnesium or calcium (Table I).

Average daily weight gains (Tables I and II) were significantly greater for the T-fed calves than for the SBO-fed calves; this response is in agreement with previous reports (13,21-23).

The tissue cholesterol was significantly greater ($P<.05$) for only the omental and intermuscular fat on the SBO-fed calves as compared with the T-fed calves (Table III); in contrast, cholesterol was significantly greater in all tissues of SBO-fed calves in a similar study in which the fat contributed 50% of dietary calories (13). The muscle, perianal fat, and coronary arteries of the SBO calves, however, also tended ($P = .09$, $P = .08$, $P = .09$, respectively) to have greater cholesterol levels than the T calves.

Liver weight as a percentage of body weight was greater in the SBO-fed than in the T-fed calves (Table II). Total liver weights and total liver cholesterol were approximately the same. The slightly greater total liver cholesterol in the T-fed calves is partly accounted for by the greater percentage liver dry matter in the T-fed calves (27.6% vs. 25.9% for the SBO-fed calves).

No differences were noted in hepatic 7 α-hydroxylase activity or in conversion rates of ^{14}C-acetate into cholesterol. No differences were noted in aortic sudanophilia between SBO- and T-fed calves.

DISCUSSION

In comparing the present study to a previous

study (13), the calves showed a profound reversal in blood cholesterol response to type of dietary fat when percentage of calories from fat was lowered from 50 to 30. A similar response to varied quantities and type of dietary fat has been noted in humans (24). However, Hegsted et al. (25) found that the amount (22% vs 40% of total calories) of dietary fat did not alter blood cholesterol in humans.

Perhaps more important than blood cholesterol response is the tissue cholesterol response. Polyunsaturated fat consumption has been shown to cause a decrease in blood cholesterol (in humans) without concurrent measurable changes in cholesterol synthesis, absorption, or excretion (7). It was proposed that cholesterol shifted out of the plasma into undefined tissue compartments. In Squirrel monkeys, a safflower oil-containing diet caused lower blood cholesterol levels than a butter-containing diet. However, whole body cholesterol was about equal in monkeys fed either fat, which indicates that cholesterol is shifted to various body pools accordng to the saturation of dietary fat (26).

In the present study, cholesterol levels were higher in adipose tissue of SBO-fed calves as compared with T-fed calves. Numerous other tissues (including muscle and liver) showed similar significant responses at the 50% calories-from-fat level; differences were in the same direction at the 30% calories-from-fat level, but were not significant at $P < .05$. Similar to the calves, growing lambs fed a safflower oil reconstituted milk have a significant increase in muscle cholesterol when compared with lambs consuming ewes' milk (27). The "increased tissue cholesterol response" that accompanies polyunsaturated fat consumption seems to vary, not only with percentage of dietary calories derived from fat, but also with maturity and (or) increased consumption of dry feed with age of the bovine (28-30). "Protected" oils fed to the mature bovine do not cause an increase in tissue cholesterol levels (29,30).

A satisfactory explanation of the often-observed plasma cholesterol-lowering properties of PUF consumption has not been formed (31). One possibility is that the decrease in blood cholesterol is accompanied by a redistribution of cholesterol in the body. Our studies and others show that, under some circumstances, dietary PUF causes an increase in tissue cholesterol concentration; whether this increased tissue cholesterol is due to a redistribution of blood cholesterol remains to be determined. It is important to know, also, whether the marked effects of type and level of fat observed in calves also occurs in man. If man responds similarly to the calf, the level of fat may be as important as the PUF/SF ratio. It would seem, further, that there may be little advantage in feeding animals (to be consumed by man) in such a way as to increase the edible tissue PUF/SF ratio if this alteration is accompanied by an increase in tissue cholesterol.

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Biosynthesis of Prostaglandins[1]

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ABSTRACT

Highly purified cyclooxygenase from sheep vesicular glands is stimulated by the presence of protoporphyrin IX compounds. This stimulation may be due to the conversion of an apoenzyme to the holoenzyme, and full activity is achieved when half of the enzyme subunits (70,000 daltons) bind heme. Also, one-half of the subunits appear to contain non-heme iron. The apparent molecular weight of the holoenzyme is approximately 300,000 daltons and is compatible with a complex of four 70,000 dalton subunits. Thus, we suggest that heme and non-heme iron may be attached to different 70,000 daltons subunits that make up an A_2B_2-type of peptide chain arrangement.

INTRODUCTION

In the overall scheme of prostaglandin formation, free fatty acid is first converted to an endoperoxide, PGG, which is then converted to other biologically active products including prostaglandins, thromboxanes, and other oxidized derivatives. The fatty acid cyclooxygenase which adds two molecules of O_2 to substrate fatty acid and forms the cyclic endoperoxide is a unique dioxygenase of central importance. A monooxygenase would utilize O_2 by adding one oxygen atom to the substrate and reducing the other oxygen to water. A dioxygenase commonly adds both oxygen atoms of O_2 to substrate. The cyclooxygenase, however, may be regarded as a *bis*-dioxygenase since it adds two O_2 molecules to the substrate fatty acid.

This unique enzyme has many regulatory features including hydroperoxide activation (1), glutathione peroxidase inhibition (2,3), reaction-catalyzed inactivation (2), and inhibition by antiinflammatory drugs (4). The enzyme must have some complicated mechanistic features if it is to create the proposed interactions of oxygen, activator, substrate and enzyme (5). To facilitate our understanding of this complex enzyme, some properties of the purified cyclooxygenase including interactions of subunits and prosthetic groups were examined.

MATERIALS AND METHODS

Materials

Sheep vesicular glands were generously donated by Upjohn Co., Kalamazoo, MI and the Flurbiprofen-Sepharose was prepared by Dr. W.L. Smith (6). Tween-40, DEAE cellulose, sodium diethyldithiocarbamate, hemoglobin, myoglobin, bovine serum albumin, catalase, phosphorylase a, and hemin were purchased from Sigma Chemical Co., St. Louis, MO. Lactate dehydrogenase was purchased from Calbiochem, (Elk Grove Village, IL,) and all other protein standards and the Blue Dextran 2000 were purchased from Pharmacia, Piscataway, NJ, Ampholine carrier electrolytes from LKB, Rockville, MD, adenosine-5′-diphosphate from P-L Biochemicals, Inc., Milwaukee, WI, and flufenamic acid from Aldrich Chemical Co., Milwaukee, WI.

Experimental Procedures

Purified cycloxygenase was obtained by following the procedures of Hemler et al. (7) in which the final enzyme material was chromatographed on Biogel P-30 or Sephadex G-200, or used immediately after isoelectric focusing without further chromatography.

To perform isoelectric focusing in a narrow range of pH values, the procedures described earlier (7) for focusing at pH 5 to 8 were scaled up using a LKB 8101 Ampholine Column (440 ml). Then the active enzyme fractions (~60 mls) plus additional ampholyte (0.14 ml, pH 5-8) were refocused in a LKB 8101 (110 ml) column. A continuous glycerol density gradient was prepared from: (a) a heavy solution that contained 9 ml of glycerol and 45 ml of enzyme solution and (b) a light solution that contained 15 ml of enzyme solution and 39 ml of 0.5 mM tris HCl (pH 8.5). After about 40 hr of focusing, 2 ml fractions were collected and the pH was determined using a Radiometer Model PHM-28 pH meter.

Heme-binding experiments consisted of incubation of purified cyclooxygenase with a threefold and tenfold molar excess of hematin per

[1]This work was presented at the 1976 Fall Meeting of The American Oil Chemists' Society at which M.E.H. was an Honored Student Awardee.

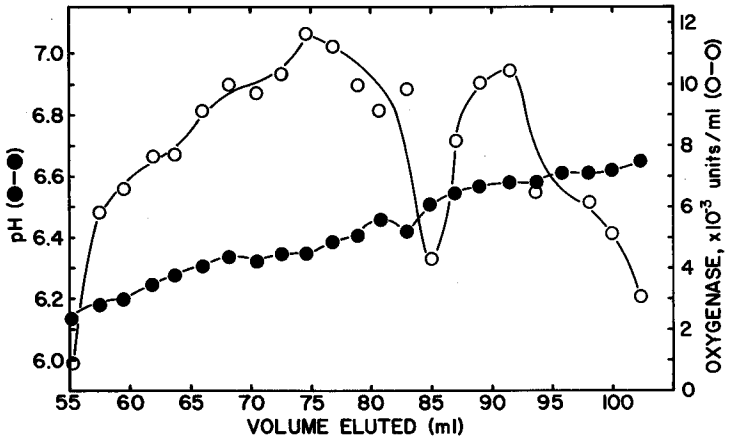

FIG. 1 Narrow range isoelectric focusing. After isoelectric focusing as described in Methods, fractions were collected and assayed for oxygenase activity (o—o) and pH (●—●).

70,000-dalton subunit for 1 hr at 0 C. The mixtures were then chromatographed on Biogel P-30 (1 x 38 cm) at 2 ml/hr at 4 C in 0.02 M sodium phosphate (pH 7.0) containing 20 % ethylene glycol. Fractions of ~1.2 ml were collected and analyzed for iron, heme, protein, and enzyme activity.

Molecular Weight Determinations

The molecular weight of cyclooxygenase subunits was determined using the SDS gel electrophoresis system described earlier (7). Protein standards of known molecular weight were included with the purified cyclooxygenase, the stained gels were scanned at 540 nm, and the position of each band was calculated relative to the bromphenol blue tracking dye. The identity of the cyclooxygenase band was verified since it was located in the region radio-labeled by [^{3}H]acetylsalicylate as previously described.

The molecular weight of active cyclooxygenase holoenzyme was estimated from gel filtration on Biogel P-300 (2.5 x 82.5 cm) and Biogel A - 1.5 m (2.5 x44 cm), each equilibrated with 0.1 M tris-chloride (pH 8.0) containing 20% ethylene glycol, 0.2% Tween-40 and 5 mM diethyldithiocarbamate. For both columns, Blue Dextran-2000 was used to calculate the void volume, and the enzyme fractions utilized had been purified up to and including the isoelectric focusing procedure.

Analytical Procedures

Iron was measured using a model AA-5 Varian Techtron atomic absorption spectrometer as described earlier (7). Protein was determined by the method of Lowry et al. (8) or by quantitative scanning of SDS gels (7). Spectra of the purified enzyme were recorded on a Cary 118 recording spectrophotometer and heme was quantitated by a modification (7) of the reduced pyridine hemochrome method of Falk (9).

Standard conditions for the assay of cyclooxygenase consisted of polarographic measurement of oxygen consumption (6,10) in reaction vessels containing 100 μM arachidonic acid, 0.5 μM bovine hemoglobin or 0.4 μM hematin, and 0.67 mM phenol in a total volume of 3 ml of 0.1 M tris-chloride (pH 8.5). Reactions were initiated by the addition of enzyme, and one unit of activity is defined as the amount of enzyme which will catalyze the uptake of 1 nmole of oxygen/min at 30 C.

RESULTS AND DISCUSSION

Isoelectric Focusing

Rome and Lands (10) previously had solubilized membrane-bound cyclooxygenase with the nonionic detergent, Tween-40, fractionated with ammonium sulfate, chromatographed on an ion exchange resin and achieved 60-fold purification. Further purification was achieved by affinity chromatography using a Flurbiprofen-Sepharose column (6) and the active fractions were then pooled and subjected to preparative isoelectric focusing (7). Results from isoelectric focusing and immunoelectrophoresis (11) experiments had suggested the possibility of multiple forms of the enzyme. Further support for this hypothesis was obtained upon performing a high resolution isoelectric focusing experiment in the range of pH 6-7. As shown in Figure 1, there appeared to be separation of enzyme into two distinguishable peaks with the approximate isoelectric points of pH 6.35 and 6.58, thus supporting the possibility

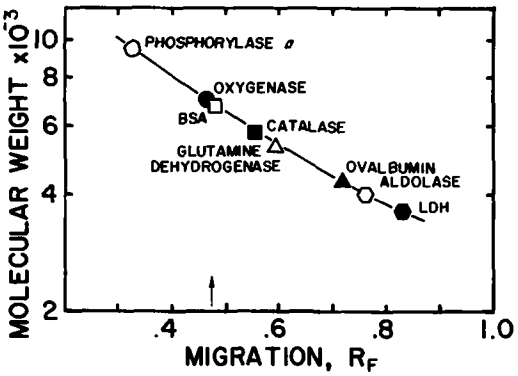

FIG. 2. Determination of molecular weight of cyclooxygenase subunits.

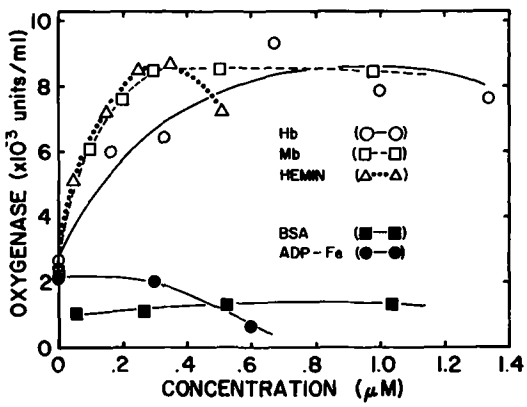

FIG. 3. Heme stimulation of cyclooxygenase activity. Varied amounts of each material were included in the otherwise heme-free standard assay mixture as indicated. For the protoheme compounds, the x-axis represents the concentration of total heme in each solution.

of two different isozymes. However, the enzyme fractions of pI~6.3 and pI~6.5 had similar specific activities (~46,000 units/mg) and visible spectra (trace peak at 412 nm). Also, the relative reactivities of the substrates, arachidonic acid and eicosatrienoic acid, were the same with both forms ($\frac{20:3}{20:4}$ = 1.5), and SDS gel electrophoresis showed that each form was composed entirely of 70,000 dalton subunits (See Fig. 2). The difference in isoelectric point may reflect an apparent charge heterogeneity, but varied amounts of bound lipid or detergent may have also contributed to multiple peak formation.

Molecular Weight

After isoelectric focusing and gel filtration, the enzyme preparation had a high degree of homogeneity as indicated by a single band upon SDS gel electrophoresis. The subunit molecular weight was determined using protein standards as shown in Figure 2. A value of 70,000 daltons/subunit was obtained in either the presence or absence of β-mercaptoethanol, reaffirming that if there are multiple subunits, they are not linked by disulfide bonds. An estimated molecular weight for the active holoenzyme was then determined from gel filtration experiments on Biogel P-300 and Biogel A-1.5 m. For each column, calculations of v_e/v_o were consistent with a molecular weight of 300,000 daltons, similar to that recently reported for the bovine enzyme (12). This value may be artificially high due to the presence of 0.2% Tween 40 in the column buffers, but it is consistent with a tetrameric arrangement of the 70,000 dalton subunits.

The Role of Heme

Previous work had shown that hematin could bind and activate cyclooxygenase which had retained only a trace of native heme after purification (7). The enzyme which forms prostaglandin in bovine seminal vesicles also seemed to be stimulated by heme compounds (12,13), and it might also have had some heme present after purification (12). The ability of several protoheme compounds to stimulate the purified sheep cyclooxygenase was examined in detail as shown in Figure 3. Upon adding increasing amounts of free hemin, or bound heme as myoglobin or hemoglobin, the basal level of activity of the purified apoenzyme was increased several fold. In control experiments, ADP-iron, a known catalyst of nonenzymatic fatty acid oxidation, had no effect, thus supporting the enzymatic nature of the heme stimulation. Also, added bovine serum albumin did not stimulate the enzyme, indicating that protein probably did not exert any nonspecific stimulatory effects.

The mechanism of cyclooxygenase stimulation by protoheme compounds may be due to conversion of the apoenzyme to a holoenzyme. A previous experiment had shown that a significant amount of heme could bind to cyclooxygenase as evidenced by a large Soret peak (7) of a heme-supplemented preparation compared to the spectrum of the apoenzyme which had only a trace peak at 412 nm. In order to more closely investigate this phenomenon, and to determine how much heme must be bound to the enzyme to attain maximal activity, purified enzyme samples were preincubated with different levels of added hematin and chromatographed to remove the free hematin.

The heme bound per 70,000 dalton subunit in three different unsupplemented preparations

TABLE I

Heme and Non-heme Iron in Cyclooxygenase[a]

Sample	Heme/70,000 daltons	Absorbance maximum (mm)	Specific activity units/mg	Stimulation by heme	Non-heme Fe/70,000 daltons
1	<0.01	412	—	—	0.63
2	0.05	412	5,143	7.7	0.81
3	0.11[b]	412	14,375	3.2	0.57[b]
4	0.49	412	18,100	1	0.75
5	1.50	409	17,250	0.8	0.37
6	2.89	406	13,750	0.8	0.42

[a]Purified unsupplemented enzyme fractions in row 1 and 2 were obtained from Biogel P-30 gel filtration experiments, and the enzyme fraction in row 3 was obtained from a G-200 column. Heme-supplemented enzyme fractions (rows 4, 5, and 6) were obtained as described in Methods. Oxidized spectra, total iron, heme concentrations, and specific activities were obtained as described in Methods. Non-heme iron levels per 70,000 daltons were calculated as total iron minus heme iron. Specific activity measurements were based on standard assay conditions without added heme. The heme stimulation is expressed as the ratio of activity obtained when heme was included in the enzyme assay mixture divided by the activity in the absence of added heme.

[b]Calculation of heme concentration for this enzyme fraction was based on an empirically derived average extinction coefficient (ϵ_{412} = 205 mM) for oxidized spectra.

TABLE II

Copurification of Non-heme Iron and Cyclooxygenase Activity[a]

Purification stage	Protein mg/ml	Units/mg protein	Total iron		Heme	
			ng/ml	nmole/mg	μM	nmole/mg
I-DEAE-cellulose	0.40	12,500	80	3.6	0.13[b]	0.3[b]
II-G-200	0.46	46,000	250	9.7	0.71[b]	1.5[b]
III-P-30	0.07	26,800	36	9.2	<0.07[c]	<1.0[c]

[a]DEAE-cellulose data were taken from tube 56 Rome and Lands (10), Figures 2 and 3, and Table III. G-200 and P-30 enzyme fractions are the same as those in Table I, row 3 and row 1, respectively.

[b]Heme concentrations were calculated using ϵ_{412} = 205 mM.

[c]Heme concentrations was determined from the reduced pyridine hemochrome, as described in Methods.

was 0.11 or less (Table I; samples 1,2,3). Analysis of the supplemented samples (4-6) showed that varied amounts of the added heme also may be bound. The preparations with low heme content (samples 1,2,3) were stimulated when assayed in the presence of protoheme compounds (as predicted from Fig. 3). On the other hand, the apparently "heme-saturated" samples (4-6) were not stimulated. Thus, it appears that 0.5 heme/70,000 daltons may be the minimum amount of that prosthetic group necessary to give optimal activity.

The maximum absorbance in the Soret region (column 3) was consistently at 412 nm when 0.5 or less heme was present per 70,000 dalton subunit. The high affinity of heme binding may be deduced from the absence of any shoulder at 370 nm that could be attributed to free heme. However, as more heme was bound to the enzyme, the optimum absorbance shifted to 409 nm (1.5 heme/subunit) and to 406 nm (2.9 heme/subunit). In the latter sample, a shoulder in the 370 nm region indicated that some loosely bound heme had dissociated to the free form. Apparently the absorbance peaks at 409 and 406 were due to heme binding at site(s) of lower affinity that are not essential for an enzyme which was already 100% active.

Presence of Non-heme Iron

Besides heme-iron, purified cyclooxygenase contains significant non-heme iron, and thus resembles another fatty acid dioxygenase, soybean lipoxygenase, which has also been shown to contain non-heme iron (14). Further evidence supporting the presence of non-heme iron is indicated by the copurification of non-heme iron and enzyme activity as shown in Table II. Rome and Lands (10), using partically purified enzyme, obtained the data shown in

row I. Our modified purification procedure, including additional steps (isoelectric focusing and gel chromtography), resulted in apparently homogenous enzyme preparations. The analyses for these preparations (II and III) show an increase in levels of iron/mg and activity/mg when compared to the cruder preparation. The lack of significant change in the apparent heme content per mg reinforces the concept that non-heme iron has co-purified with cyclooxygenase.

In order to quantitate the amount of non-heme iron in the various cyclooxygenase fractions listed in Table I, they were each assayed for total iron using flameless atomic absorption spectrometry, and the amount of heme iron was subtracted. The last column in Table I shows that a relatively constant amount of non-heme iron per 70,000 dalton subunit was obtained for each sample. There was some variability, but the average number of non-heme atoms per 70,000 daltons appears to be about 0.6. While further studies of the heme and non-heme iron stoichiometry are in progress, these preliminary numbers of 0.5 heme per subunit and 0.6 non-heme iron per subunit lead to an interesting theory. Perhaps there are two different kinds of subunits of 70,000 molecular weight, one which contains non-heme iron, and the other which binds heme. The tetrameric holoenzyme would then have an A_2B_2-type of arrangement. The combintation of heme and non-heme iron in one enzyme has no known precedent. However, the cyclooxygenase reaction appears to include two dioxygenase steps. One may be analagous to the reaction catalyzed by lipoxygenase, an enzyme containing non-heme iron (14), and there may be a heme-mediated reaction analogous to tryptophan oxygenase catalysis. The latter enzyme has two types of subunits of similar molecular weight (15) and half of the subunits appear to bind heme. Tryptophan and indoleamine oxygenase enzymes were recognized as the only heme proteins known to catalyze the dioxygenase type of reaction (16,17). Cyclooxygenase now represents a second type of dioxygenase activity that requires heme for catalysis.

With the purification and preliminary characterization reported here, the door is now open for mechanistic and regulatory insights. For example, the requirement for heme to convert the apoenzyme into a holoenzyme suggests that heme might have a very important physiological regulatory role in prostaglandin biosynthesis. Perhaps the heme must be available to the membrane-bound apoenzyme in order to convert it to the active form, and agents which would interfere with heme synthesis, availability, or binding could regulate prostaglandin synthesis.

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Changes in the Fatty Acid Composition of Rat Lung Lipids During Development and Following Age-Dependent Lipid Peroxidation

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ABSTRACT

Analyses of the fatty acid content and composition of various lung lipids were conducted in rats 1 day, 5 days, and 12 days after birth and in adult animals in order to define more clearly the specific lipid peroxidizing system found in neonatal rat lungs. Lipid peroxidation occurs in the 900 x g supernatant fraction of rat lung homogenates in an age-dependent manner independent of the addition of any factor and is maximal at 5 days of age. No lipid peroxidation is evident in similar preparations of either newborn or adult lung tissue. As the animals develop, arachidonic and docosahexaenoic acids, fatty acids which are both highly susceptible to lipid peroxidation in the presence of a suitable catalyst, decrease gradually when measured as the percentage of the total fatty acids in the triglyceride fraction of the lung. The total quantity of triglycerides, however, is significantly lower in lungs from 1-day-old rats than at any other age. The fatty acid composition and total quantity of both lung phospholipids and lung free fatty acids do not show similar changes. Following in vitro incubation of the 900 x g supernatant fraction of peroxidizing lung homogenates, an appreciable decrease in the amount of arachidonic and docosahexaenoic acid could be detected in lung triglycerides. Less extensive decreases were observed in the phospholipid fraction. No changes in these components were observed in newborn or adult animals. The addition of triarachidonin to the 900 x g supernatant fraction of lung homogenates resulted in increased malondialdehyde release at all ages tested while added arachidonic acid increased the formation of malondialdehyde only in 5- and 12-day-old rat lung preparations. The addition of triolein, cholesterol arachidonate, and diarachidonyl phosphatidylcholine had no effect on malondialdehyde formation at any age. The age-dependent lipid peroxidation observed after in vitro incubation of rat lung homogenate preparations, therefore, may result from the relatively high concentration of triglycerides containing polyunsaturated fatty acids present in the neonatal tissue. As the susceptible polyunsaturated fatty acids of lung triglycerides are replaced by less unsaturated species, this activity may diminish concomitantly.

INTRODUCTION

Lipid peroxidation, a reaction studied extensively in both microsomal and mitochondrial fractions prepared from a variety of tissues (1), requires a transition metal ion for initiation, and the presence of either NADPH or ascorbate for the enzymatic or the nonenzymatic reaction, respectively (2). Furthermore, the polyunsaturated fatty acids arachidonate and docosahexaenoate which are substrates for the NADPH-catalyzed microsomal lipid peroxidation are localized primarily in the microsomal phospholipids (3,4).

In this laboratory, an age-dependent, heat labile, lipid peroxidizing activity was shown to occur in neonatal rat lung homogenates without the addition of any factor (5). No concomitant deficiency of either glutathione peroxidase, glutathione reductase or α-tocopherol, factors which have been postulated to protect membrane lipids from oxidation (2,6,7), was observed in the susceptible age group (5). An age-related alteration in the quantity of polyunsaturated fatty acids in neonatal rat lungs, however, might provide the basis for age-dependent pulmonary lipid peroxidation. A similar compositional change was invoked to explain reported age-related alterations in lipid peroxidation in rat liver mitochondria (8). Previous studies on the fatty acid composition of triglycerides and phospholipids in developing rat lungs did not include complete information on the content of arachidonic and docosahexaenoic acids (9), which are highly suscepti-

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ble to peroxidation and subsequent oxidative cleavage to form malondialdehyde (10). This study, therefore, provides more complete information on the fatty acid composition of the total lipids, phospholipids, and triglycerides, in the adult as well as the developing rat lung. Furthermore, the fatty acid composition of triglycerides and phospholipids in the 900 x g supernatant fraction of lung homogenates was examined both before and after incubation at 37 C. Under these experimental conditions, lipid peroxidation occurs during the susceptible age period. Exogenous phospholipids, triglycerides, free fatty acids and cholesterol esters have also been added to these lung preparations to confirm the specific class of lipids susceptible to this age specific peroxidation.

MATERIALS AND METHODS

Adult Sprague-Dawley rats were obtained from Bio Lab (St. Paul, MN) and bred in our laboratory as described elsewhere (11). Lung tissue was obtained from 1-day-old, 5-day-old, 12-day-old, and adult (250-350 g) rats of both sexes. The animals were decapitated and the lungs perfused with 0.9% NaCl. The lungs were then excised and placed in cold potassium phosphate buffer (pH 7.8, 0.05 M). Pooled tissue was homogenized (1:4, w/v) in a Sorvall Omni-Mixer in the same buffer. Lipids were extracted by the procedure of Folch et al. (12). Lipid peroxidation experiments were carried out with the 900 x g supernatant fraction of lung homogenates by incubation of 2 ml samples at 37 C in a shaking water bath under a gassing hood filled with flowing air. Malondialdehyde formation was used as an indicator of lipid peroxidation and was measured in 100 μl aliquots using the thiobarbituric acid assay (13). Lipid peroxidation in this system has been confirmed by methods additional to malondialdehyde formation (5). Protein concentrations were measured by the microbiuret method (14).

Phospholipids and triglycerides in the lipid extracts were isolated using thin layer chromatography (15). The thin layer plates were developed in chloroform-hexane (75:25, v/v) followed by chloroform-methanol (95:5, v/v). The areas of the plate corresponding to the phospholipids and the triglycerides were scraped and extracted with methanol and chloroform, respectively. The fatty acids in the total lipid extract, the phospholipids, and the triglycerides were transesterified using BF_3 (14%) in methanol (16) to form the methyl ester derivatives. The fatty acid methyl esters were quantitated on a Hewlett-Packard model 5830A gas chromatograph equipped with dual flame ionization detectors using methyl linolenate as the internal standard. The methyl esters were separated on a 6 ft glass column packed with 10% SP-2340 on 100-120 chromosorb W AW (Supelco, Bellefonte, PA), time programming from 175 C to 215 C at 4 C per min following an initial 8 min holding period at 175 C. Individual fatty acid methyl esters were identified by comparing retention times with standards obtained from Analabs (North Haven, CT) or Applied Science (State College, PA).

Triarachidonin was obtained from Analabs, triolein from Applied Science, arachidonic acid, cholesterol arachidonate and BF_3 (14%) in methanol from Sigma (St. Louis, MO) and L-3-phosphatidylcholine diarachidonyl from Serdary Research Laboratories, (London, Ontario). All other chemicals used were of reagent grade.

RESULTS

We have previously reported that an age-dependent lipid peroxidizing activity appeared in rat lung homogenates 2 days following birth, rapidly increased to a maximum at 5 days after birth and then began to decline until by 20 days after birth no malondialdehyde release could be detected (5). Fatty acid analyses were conducted on the total lipid extract, the triglycerides and the phospholipids from 1-day-old, 5-day-old, 12-day-old and adult rat lungs. Of all the polyunsaturated fatty acids identified in the lung, arachidonic acid and docosahexaenoic acid are present in the greatest quantity (Table I). Both have been shown to be highly susceptible to lipid peroxidation and to decompose to form malondialdehyde (2,10). Table I also shows that as the lungs developed, these two fatty acids decreased from 28.2% of the total fatty acids in 5-day-old rats, the age at which maximal lipid peroxidation occurs, to 19.3% in adult rat lungs. This change was confined to the triglyceride fraction where these polyunsaturated fatty acids decreased from 31.1% of the total fatty acids in 5-day-old rat lungs to 4.1% in adults. Arachidonate and docosahexaenoate did not change with age in lung phospholipids. The content of oleic and linoleic acids, however, increased from 36.3% of the total triglyceride fatty acids in 5-day-old rat lungs to 59.3% in the adults. Similar to the age-dependent change in polyunsaturated fatty acids, this increase was observed only in the triglyceride fraction. The phospholipids showed no age-dependent changes in the composition of these fatty acids (Table I). The free fatty acids, present at only 20% of the phospholipid

TABLE I

Fatty Acid Composition of Rat Lung Lipids[a]

Fatty acid	Total fatty acids				Triglycerides				Phospholipids			
	1 Day	5 Day	12 Day	Adult	1 Day	5 Day	12 Day	Adult	1 Day	5 Day	12 Day	Adult
Laurate 12:0	1.5	1.5	1.7	0.6	–	0.7	1.9	–	–	–	–	–
Myristate 14:0	2.4	3.8	4.4	2.2	1.3	2.4	4.2	1.9	1.7	2.9	2.3	1.4
Myristoleate 14:1	3.2	3.0	3.6	3.0	0.5	0.4	0.5	0.4	5.1	6.0	6.7	8.2
Palmitate 16:0	29.1	24.5	24.9	27.7	18.5	21.0	24.1	22.1	26.7	20.9	22.2	24.5
Palmitoleate 16:1	5.9	2.0	2.6	5.3	3.4	2.2	2.3	5.8	5.0	2.0	2.2	3.7
Stearate 18:0	9.6	10.3	10.5	11.2	7.5	6.1	7.4	6.4	10.6	12.8	12.6	12.9
Oleate 18:1	15.8	17.9	16.7	20.1	29.1	22.1	24.0	37.6	14.2	14.3	13.5	12.5
Linoleate 18:2	5.1	8.9	9.8	10.6	12.2	14.1	16.8	21.7	4.1	5.8	6.6	5.7
Arachidonate 20:4	18.9	17.4	16.2	15.0	12.7	9.7	6.9	2.2	24.7	26.2	25.7	24.9
Docosahexaenoate 22:6	8.5	10.8	9.6	4.3	14.8	21.4	12.0	1.9	7.9	9.1	7.3	6.3

[a]Values are expressed as the percent by weight of the fatty acid methyl esters measured in lipid extracts of lung homogenates and are the average of three determinations.

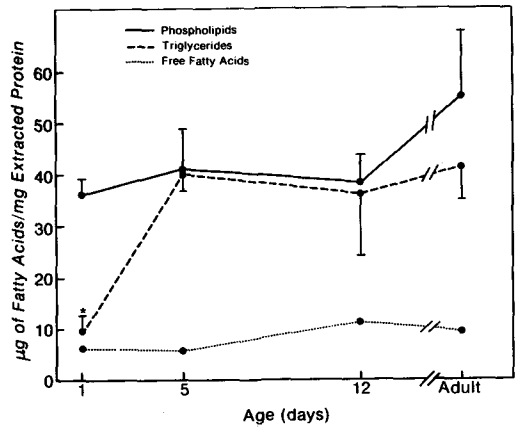

FIG. 1. Lipids were extracted from lung homogenates isolated by thin layer chromatography and the fatty acids assayed by gas chromatography. Results are reported per mg of protein determined before lipid extraction. Values for the phospholipids and the triglycerides are the average of three determinations ± SEM. The values for the free fatty acids were obtained from one determination at each age. *Significantly lower than at other ages ($p<0.05$).

fatty acids (Fig. 1), contain less than 1% arachidonate and docosahexaenoate and did not fluctuate with age (data not shown). Other lipids such as monoglycerides, diglycerides, and cholesterol esters constitute only a minor portion of lung lipids (17) and therefore were not analyzed for their fatty acid composition.

Arachidonate and docosahexaenoate constitute 27.4% of the total fatty acids in 1-day-old rat lungs and 28.2% in 5-day-old rat lungs (Table I). Figure 1 illustrates that, although these percentages are approximately equal, the total quantity of fatty acids per mg of protein from the triglyceride fraction of 1-day-old rat lungs is significantly less than that at any other age ($p<0.05$) while the total quantity per mg of protein from the phospholipid and free fatty acid fractions remains constant ($p>0.2$). Analyses of the changes in the quantity of individual fatty acids in the triglyceride fraction are shown in Figure 2. It is evident that the changes in the quantity of arachidonic acid plus docosahexaenoic acid are similar to the age-dependent changes seen with malondialdehyde release from lung homogenates (5). The maximum quantity of polyunsaturated fatty acids in the triglyceride fraction occurs at 5 days of age where maximal malondialdehyde release is observed.

Lipid peroxidation analyses were conducted on the supernatant fraction of rat lung homogenates obtained after centrifugation at 900 x g. The same age-dependence as previously reported (5) was observed. Extraction of this

supernatant fraction for lipids and an analysis of the fatty acids both before and after incubation at 37 C for 90 min revealed no change in the amount of palmitic acid recoverable in either the phospholipid or the triglyceride fraction. Therefore, the data in Tables II and III are expressed as the percent by weight of palmitate. This method allows changes in the fatty acid composition following lipid peroxidation to be more readily discerned (3). Changes in lung triglycerides were observed in the arachidonate and docosahexaenoate content at 5 and 12 days of age where these two fatty acids showed a total decrease of 32.2% following lipid peroxidation in 5-day-old rat lungs and 9.8% in 12-day-old lungs (Table II). Decreases were also seen with arachidonate and docosahexaenoate in the phospholipid fraction (Table III). In this fraction, the decrease totaled 14.5% following lipid peroxidation in both 5- and 12-day-old rat lungs. The triglyceride and the phospholipid fractions showed little change in fatty acid composition following incubation of the 900 x g supernatant fraction from either 1-day-old or adult lung homogenates.

The data obtained from the fatty acid analyses of rat lung lipids described above suggest that both the triglycerides and the phospholipids could be the source of the fatty acids utilized as the substrate for this age-dependent lipid peroxidizing activity. To aid in the identification of the specific lipid class which actually provides peroxidizable fatty acids, various lipids were added to a 900 x g supernatant fraction from 1-day-old, 5-day-old, 12-day-old and adult lung homogenates. The effect of these lipids on malondialdehyde release following a 30 min incubation is reported in Table IV. It is evident that following a 30 min incubation, triarachidonin was capable of stimulating malondialdehyde release in lung preparations from all ages tested, while triolein and diarachidonyl phosphatidylcholine were without effect (Table IV). Arachidonic acid augmented malondialdehyde release only at 5 and 12 days of age. The effect of cholesterol arachidonate, although small, appears to be similar to that of arachidonic acid. When these lipids were incubated in buffer alone at the same concentrations present in the lung preparations, the maximum quantity of malondialdehyde formed was less than 5% of the amount released in the presence of the lung fraction indicating that autooxidation could not explain the effect.

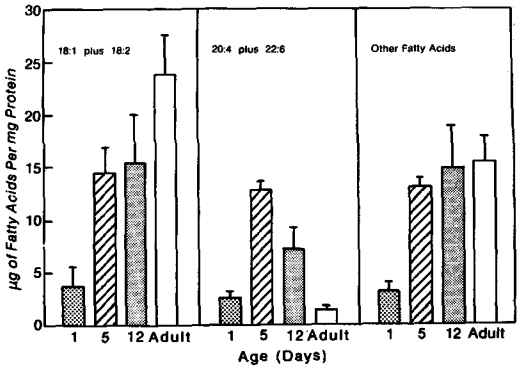

FIG. 2. The quantity of fatty acids per mg of protein in lung triglycerides as a function of age. Lipids were extracted from lung homogenates, the triglycerides isolated by thin layer chromatography and the fatty acids assayed by gas chromatography. Results are reported per mg of protein determined before lipid extraction. The data for oleic plus linoleic acids, arachidonic plus docosahexaenoic acids and the remaining fatty acids are combined into three separate groups.

DISCUSSION

It has been reported by Barber and Wilbur that tissues most susceptible to lipid peroxidation appear to be those with low mitotic rates such as brain, liver, and kidney, while rapidly multiplying tissues such as the intestinal epithelium or testes are resistant (18). Neonatal rat lungs, however, undergo rapid growth, cellular proliferation and differentiation throughout the age period in which lipid peroxidation of lung preparations occurs (5,19,20). Previous reports have indicated that those fatty acids subject to lipid peroxidation are found primarily in the tissue phospholipids (3,4), although neutral lipids have also been identified as substrates (21). Alterations in the fatty acid composition of lung phospholipids but not the triglycerides have been reported to occur in response to hyperoxia (22). The fatty acid analyses reported in this study suggest that it is the component fatty acids of the triglycerides and not of the phospholipids in the lung which are altered during the sensitive age period and that it is the triglycerides which are the primary source of the fatty acids which are peroxidized. The data obtained from the addition of exogenous lipids further supports this concept. The high degree of age-dependence observed with this lipid peroxidizing activity in neonatal rat lungs appears to be due to the combined effect of an increasing quantity of lung triglycerides during development and the replacement of the polyunsaturated fatty acids with less unsaturated species in the triglycerides as maturation progresses.

These changes seen in the fatty acid composition of lung lipids are similar to those seen in liver lipids during development of the rat

TABLE II

The Effect of Lipid Peroxidation on the Fatty Acid Composition of Rat Lung Triglycerides[a]

Fatty acid		1 Day		5 Day		12 Day		Adult	
		Preincubation	Percent change	Preincubation	Percent change	Preincubation	Percent change	Preincubation	Percent change
Laurate	12:0	–	–	2.4 ± 1.2	+1.1	5.8 ± 2.4	−0.1	–	–
Myristate	14:0	5.4 ± 0.4	−0.2	9.3 ± 1.4	+1.6	15.1 ± 3.1	+0.3	8.6 ± 0.4	+1.6
Myristoleate	14:1	0.4 ± 0.4	+0.1	1.6 ± 0.2	+0.5	1.8 ± 0.2	0.0	1.4 ± 0.5	+0.7
Palmitoleate	16:1	15.1 ± 0.8	−3.1	12.3 ± 3.0	+1.9	8.0 ± 1.2	+0.2	26.2 ± 1.4	+1.8
Stearate	18:0	35.3 ± 0.7	+1.9	29.5 ± 3.4	−1.7	28.8 ± 0.9	−0.6	28.1 ± 0.9	−3.3
Oleate	18:1	156.8 ± 8.8	+9.3	114.3 ± 7.0	−4.4	94.8 ± 3.0	+0.9	172.1 ± 10.9	−17.3
Linoleate	18:2	56.8 ± 3.5	+0.4	71.9 ± 4.9	−5.9	68.6 ± 4.1	−2.2	94.0 ± 8.8	−12.9
Arachidonate	20:4	71.0 ± 5.2	−2.0	49.7 ± 3.1	−7.3	31.5 ± 2.2	−2.0	8.1 ± 0.8	−1.3
Docosahexaenoate	22:6	89.7 ± 27.2	+7.1	121.7 ± 17.7	−24.9	69.0 ± 2.5	−7.8	8.6 ± 0.8	−2.0

[a]Triglycerides were isolated by thin layer chromatography from a lipid extract of the 900 x g supernatant fraction of a rat lung homogenate before and after incubation at 37 C for 90 min. The data are expressed as the percent by weight of palmitate and are the average of three determinations ± SEM.

TABLE III

The Effect of Lipid Peroxidation on the Fatty Acid Composition of Rat Lung Phospholipids[a]

Fatty acid		1 Day		5 Day		12 Day		Adult	
		Preincubation	Percent change	Preincubation	Percent change	Preincubation	Percent change	Preincubation	Percent change
Myristate	14:0	6.3 ± 0.2	−0.2	12.2 ± 2.2	+0.1	16.2 ± 1.4	+0.5	8.9 ± 1.4	+0.2
Myristoleate	14:1	9.2 ± 0.1	−0.1	14.3 ± 1.8	+0.1	19.5 ± 3.7	+2.7	11.1 ± 1.5	+2.7
Plamitoleate	16:1	17.9 ± 1.1	−0.2	9.1 ± 1.0	+0.8	8.3 ± 0.4	+1.3	18.5 ± 0.9	−1.5
Stearate	18:0	25.9 ± 0.8	+0.8	45.7 ± 4.6	−4.8	54.9 ± 1.9	−1.9	34.1 ± 1.0	−0.9
Oleate	18:1	37.4 ± 1.0	+2.1	52.2 ± 4.0	−3.1	57.4 ± 0.3	+1.0	34.3 ± 1.8	−1.0
Linoleate	18:2	10.6 ± 0.7	−0.6	23.1 ± 2.1	−1.6	30.5 ± 0.6	−1.3	20.0 ± 1.0	−0.6
Arachidonate	20:4	64.5 ± 1.8	+1.7	86.3 ± 8.2	−10.2	102.1 ± 4.4	−9.8	62.9 ± 3.6	−2.9
Docosahexaenoate	22:6	19.9 ± 2.9	+0.1	29.1 ± 1.9	−4.3	29.1 ± 0.5	−3.7	16.1 ± 0.8	−0.8

[a]Phospholipids were isolated by thin layer chromatography from a lipid extract of the 900 x g supernatant fraction of a rat lung homogenate before and after incubation at 37 C for 90 min. The data are expressed as the percent by weight of palmitate and are the average of three determinations ± SEM.

TABLE IV

The Effect of Added Lipids on Malondialdehyde Formation in Rat Lung Homogenate Preparations[a]

Additions[b]	Concentration	1 Day	5 Days	12 Days	Adult
None	–	0.30 ± 0.05	1.70 ± 0.33	1.45 ± 0.24	0.36 ± 0.02
Triarachidonin[c]	50 μg/ml	0.13 ± 0.04	0.64 ± 0.17	0.37 ± 0.20	0.26 ± 0.08
Triarachidonin	100 μg/ml	0.25 ± 0.06	0.60 ± 0.13	0.85 ± 0.22	0.60 ± 0.05
Triarachidonin	200 μg/ml	0.61 ± 0.26	1.62 ± 0.33	1.37 ± 0.25	1.05 ± 0.07
Triarachidonin	300 μg/ml	0.65 ± 0.18	1.39 ± 0.17	1.61 ± 0.18	1.69 ± 0.15
Triolein	200 μg/ml	−0.06 ± 0.01[d]	0.10 ± 0.21	−0.11 ± 0.06[d]	−0.08 ± 0.03[d]
Diarachidonyl phosphatidylcholine[c]	200 μg/ml	0.05 ± 0.06	−0.23 ± 0.21[d]	−0.08 ± 0.44[d]	0.09 ± 0.04
Arachidonic acid	200 μg/ml	0.04 ± 0.04	0.90 ± 0.23	1.04 ± 0.22	0.01 ± 0.04
Cholesterol Arachidonate	200 μg/ml	0.01 ± 0.02	0.33 ± 0.23	0.36 ± 0.16	0.14 ± 0.03

[a]Lipids were added to the 900 x g supernatant fraction of a rat lung homogenate. Malondialdehyde formation was measured following a 30 min incubation at 37 C. The data are expressed as micromoles of malondialdehyde per mg of protein formed above the control without added lipid. An extinction coefficient of 1.56 x 10^5 $M^{-1}cm^{-1}$ was used for the determination of malondialdehyde concentrations.

[b]The lipids were suspended in potassium phosphate buffer, pH 7.8, 0.05 M, at a concentration of 2 mg/ml and sonicated for 10 sec before addition to the incubation mixture. Malondialdehyde formation, in an equal sample volume, following incubation of these lipids in buffer alone at 37 C for 30 min was less than 5% of that observed in the presence of the lung fractions.

[c]From gas chromatographic analyses the triarachidonin contained 92% arachidonic acid and the diarachidonyl phosphatidylcholine 86%.

[d]Negative values mean less malondialdehyde was formed in the presence of added lipid than in the control.

(23). Rat milk, the only source of nourishment for neonatal rats up to 30 days of age, contains large quantities of triglycerides (24) and it has been suggested that the rapid increase in the lipid content of various organs after birth is due to the high fat content of the milk (9,23). Although this may explain the increase in the total quantity of triglyceride in rat lungs, it does not explain why the lung should expend the energy to accumulate triglycerides containing long chain polyunsaturated fatty acids from precursors present in rat's milk (23,24). It has been reported that the polyunsaturated fatty acids in rat liver triglycerides are derived directly from the rat's milk rather than by synthesis from precursors (23). This may also occur in the lung and could have some functional significance relating to the metabolic requirements of cellular growth, differentiation, and lung maturity.

If the quantity of triglyceride present in the lung is important in the development of this age-dependent lipid peroxidizing activity, one would expect alterations in the quantity to alter the amount of malondialdehyde released. This appears to be the case since 1-day-old rat lungs, which release little malondialdehyde, contain significantly lower amounts of triglyceride compared to rat lungs at other ages. Furthermore, the addition of a triglyceride such as triarachidonin, which contains fatty acids susceptible to lipid peroxidation, to 1-day-old rat lungs will enhance malondialdehyde formation indicating that 1-day-old rat lungs are capable of peroxidizing these lipids. It is known that fasting will decrease the lung lipid content (25). We have observed that lungs from 5-day-old rats which were poorly fed by their mothers failed to produce appreciable amounts of malondialdehyde. This again suggests that age-dependent lipid peroxidation in neonatal rat lungs may be due to an increase in the quantity of pulmonary triglycerides which contain polyunsaturated fatty acids. As the rat lung continues to develop, the quantity of triglyceride remains relatively constant while the content of polyunsaturated fatty acids begins to decrease. Since these less unsaturated species are less susceptible to lipid peroxidation and will not decompose to form malondialdehyde (2,10), the amount of measurable malondialdehyde released by lung homogenates begins to decline as the animal grows older. The addition of triarachidonin will again result in the formation of malondialdehyde by the lung preparation indicating that lung preparations from rats of any age are capable of peroxidizing lipids if the appropriate substrate is available.

Since arachidonic acid was capable of augmenting malondialdehyde release with the same age-specificity as the endogenous lipid peroxidizing activity, it is possible that free fatty acids and not triglycerides are the endogenous source of the substrate for age-dependent lipid peroxidation. This is judged to be unlikely, however, since the quantity of peroxidizable free fatty acids in the tissue was below the level of detection by gas chromatographic analysis. Furthermore, although the addition of arachidonic acid to 1-day-old or

adult lung fractions failed to produce any malondialdehyde, these fractions were capable of peroxidizing the same fatty acids contained in triglycerides. The rapid increase in the quantity of triglycerides in the lung from 1 to 5 days of age suggests that neonatal lungs rapidly develop the capacity to synthesize triglycerides. The age-specificity for enhanced malondialdehyde formation seen with added arachidonic acid may therefore be caused by the development of the ability to synthesize triglycerides containing polyunsaturated fatty acids followed by a decline in this ability as maximum lung growth is achieved.

In spite of the fact that lipid peroxidation is considered to be a manifestation of tissue damage in many biological systems (26,27,28) and has been implicated in pathological processes such as pulmonary oxygen toxicity (29) and aging (30), the ability of neonatal lung fractions to peroxidize lipids in vitro appears to have no obvious effect on their in vivo development. Indeed, neonatal rats are extremely resistant to the toxic effects of oxygen when compared to adult rats (31), thus supporting evidence that lipid peroxidation may not be related to oxygen toxicity (1). Clements (32) has suggested that the resistance which poikilotherms exhibit toward oxygen toxicity may be due to the high content of polyunsaturated fatty acids in the neutral lipids and phospholipids of their lungs. It is possible, therefore, that the high content of polyunsaturated fatty acids in the triglycerides from lungs of neonatal rats may contribute to their resistence to oxygen toxicity.

Lipid peroxidation is generally believed to be a process involving the lipids of biological membranes (1). Triglycerides are known to constitute only a minor portion of membrane lipids (33). This peroxidizing activity in neonatal rat lungs appears to utilize triglycerides as the primary source of fatty acids undergoing peroxidation and can be measured in a 105,000 x g neonatal lung supernatant fraction suggesting that this age-dependent lipid peroxidation is probably not a membrane associated phenomenon. Rather, this activity may indicate the quantity of polyunsaturated fatty acids in lung triglycerides and may provide information on the metabolism of the developing lung.

The relationship between lung growth and differentation and the metabolic demands of the tissue is complex. The lung consists of over 40 different cell types (34). Type I and type II pneumocytes and pulmonary fibroblasts, which comprise 80% of all lung cells (35) show maximum rates of proliferation at the time of both maximum malondialdehyde formation and maximum content of polyunsaturated fatty acids. It is possible that these changes in lung fatty acids and age-dependent lipid peroxidation may reflect a shift in the cellular population of the lung and the metabolic demand of the maturing lung. Human lung, like rat lung, is relatively immature at birth (20). These experiments, therefore, may shed some light on the biochemical parameters of human lung development and the associated alterations in metabolic status.

ACKNOWLEDGMENTS

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The Effect of Long-Chain Monoenes on Prostaglandin E_2 Synthesis by Rat Skin[1]

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ABSTRACT

In order to ascertain whether the dermal lesions observed in male rats fed rapeseed oils are due to impaired prostaglandin biosynthesis, endogenous levels of prostaglandin E_2 (PGE_2) in skin and the capacity of this tissue to synthesize PGE_2 from arachidonic acid was investigated. Male Sprague-Dawley rats were fed from weaning for 8 weeks either a standard rat diet (chow) or semisynthetic diets containing 20% by weight of the following fat sources: corn oil; commercial lard; commercial lard to which was added 5.4% free erucic acid; rendered pig fat; or the following rapeseed oils: *Brassica napus* var. Zephyr; *B. campestris* var. Span; *B. campestris* var. Arlo (15%) and var. Echo (85%) designated HEAR (high erucic acid rapeseed). The long-chain monoene content (18:1, 20:1, and 22:1) of the diets fed ranged from 30 to 71 mole % and that of skin from 27 to 74 mole %. A significant ($P < 0.01$) correlation was found between the level of 18:2n-6 in the diet and the endogenous PGE_2 levels in skin and the capacity of this tissue to synthesize PGE_2. No relationship was found between these two PGE_2 parameters and the level of erucic acid in the diet. The rate of turnover of PGE_2 appeared to be lower in rats fed rapeseed oil as evidenced by the relatively high endogenous PGE_2 levels when these oils were fed (96 to 105 μg/g). On the other hand, the lowest capacity for PGE_2 synthesis was found with skin from rats fed Zephyr rapeseed oil, rats which also had the most severe incidence of hair loss and dermal lesions. Significant ($P < 0.01$) negative correlations were observed between the level of monoenes and specifically the level of oleic (18:1n-9) acid in the diets and PGE_2 synthesis capacity of skin, possibly confirming the known inhibitory effect of 18:1n-9 on the prostaglandin synthesizing enzyme system.

INTRODUCTION

When male rats were fed fully refined rapeseed oil, up to 50% of the animals developed ermal lesions on the feet and tail and showed evidence of alopecia (1), similar to that described in the case of an essential fatty acid (EFA) deficiency (2).

Van Dorp et al. (3) first suggested that the signs of essential fatty acid deficiency may be due to a decreased synthesis of prostaglandins, and later demonstrated (4) that the skin of EFA deficient rats is almost devoid of prostaglandins. Ziboh and Hsia (5) demonstrated that the lesions caused by EFA deficiency could be cured (healed) by topical application of prostaglandin E_2 (PGE_2) to the affected areas.

Carney et al. (6) reported that the stimulation of prostaglandin production by adrenocorticotrophin to be considerably lower in adrenals from rats fed rapeseed oil than in rats fed corn oil.

In view of the dermal lesions and alopecia observed with rats fed rapeseed oils and the fact that prostaglandins are formed in situ, the present study was initiated to investigate the capacity of the skin of these animals, a tissue normally rich in prostaglandin synthetase (7), to produce PGE_2. The effect of feeding erucic acid in the free or esterified form on PGE_2 synthesis was also studied.

MATERIALS AND METHODS

The following oils were used: corn oil; commercial lard; commercial lard to which was added 5.4% free erucic acid (L + EA); rendered pig fat (RPF–see ref. 8); *Brassica napus* var. Zephyr rapeseed oil; *B. campestris* var. Span rapeseed oil; *B. campestris* var. Arlo (15%) and var. Echo (85%) designated HEAR (high erucic acid rapeseed) oil. All rapeseed oils were processed and stored as previously described (9).

Male Sprague-Dawley rats, 45 to 50 g, were purchased (Bio-Breeding Laboratories, Ottawa, Canada) at weaning (3 wk), and fed a semisynthetic diet (1) to which was added 20% by weight test oil. One additional group of rats was fed laboratory rat chow (Ralston Purina, Woodstock, Ontario). Rats were randomly assigned, two per cage and four per diet treat-

[1]Contribution No. 687, Animal Research Institute.

TABLE I

Fatty Acid Composition of the Diets

Fatty acid[a]	Diets[b]							
	Chow	Corn	Lard	L + EA	RPF	Zephyr	Span	HEAR
16:0	16.9	10.6	25.6	22.4	12.6	5.8	4.8	3.1
16:1	1.8	0.1	2.6	3.1	2.2	0.2	0.2	0.2
18:0	3.2	1.8	14.6	14.7	4.8	2.6	2.1	1.5
18:1n-9	22.4	25.7	43.2	37.9	39.1	65.2	58.6	34.9
18:2n-6	38.9	60.3	9.6	10.4	18.5	17.5	19.5	16.6
18:3n-3	3.1	0.9	0.6	0.7	6.1	5.6	5.2	5.6
20:1	2.9	0.2	1.2	1.4	8.3	1.3	3.3	12.0
22:1	3.7	0.1	0.2	5.6	5.6	0.6	4.8	23.6
Monoenes	30.8	26.1	47.2	48.0	55.2	67.3	66.9	70.7

[a]Fatty acid composition is expressed as mole % of total fatty acids; fatty acids are designated by number of carbon atoms:number of double bonds.

[b]Chow = Purina Laboratory Rat Chow; Corn = corn oil; Lard = commerical lard; L + EA = commercial lard + 5.4% free erucic acid; RPF = rendered pig fat [see Hulan et al. (8)]; Zephyr = *Brassica napus* var. Zephyr rapeseed oil; Span = *B. campestris* var. Span rapeseed oil; HEAR = *B. campestris* var. Arlo (15%) and var. Echo (85%) rapeseed oil.

TABLE II

Fatty Acid Composition (mole %) of the Rat Skin Used in this Study

Fatty acid[a]	Diets[b]							
	Chow	Corn	Lard	L + EA	RPF	Zephyr	Span	HEAR
16:0	32.7	25.4	32.0	31.8	19.4	11.6	13.3	13.4
16:1	4.0	1.5	0.4	0.9	1.2	0.8	1.9	1.5
18:0	5.6	10.0	8.0	10.2	5.1	2.5	2.1	4.2
18:1n-9	33.8	25.6	50.3	48.0	54.2	69.3	68.5	47.7
18:2n-6	18.6	32.5	6.7	5.1	10.0	12.0	9.9	9.9
18:3n-3	0.4	0.3	0.1	0.1	0.5	1.3	0.7	1.8
20:1	2.3	0.4	0.7	1.5	6.6	1.1	2.5	9.1
20:4n-6	0.3	0.2	0.2	0.2	0.1	0.2	0.1	0.1
22:1	0.4	–	–	1.0	1.3	0.2	0.7	5.3
Monoenes	40.5	27.5	51.4	51.4	63.3	71.4	73.6	63.6

[a]See footnote a, Table I.

[b]See footnote b, Table I.

ment, and maintained on the different treatments for 8 wk at which time all animals were killed. The skin was immediately removed, frozen on dry ice, and stored at −15 C for subsequent lipid (fatty acid) analysis and prostaglandin assay. Details of the lipid analyses have been published elsewhere (1).

Samples of skin were prepared for prostaglandin assay using the ground powder technique described by Tan and Privett (10). For the conversion of arachidonic acid to PGE_2, incubations were carried out in an oxygen atmosphere for 20 min at 37 C using 10 ml of the same incubation media as described by Pace-Asciak and Wolfe (11). at pH 7.9. For each determination, 500 mg of ground powder and 0.33 mM of 5,8,11,14-eicosatetraenoic acid (Grade 1, arachidonic acid, ca. 99% pure, Sigma Chemical Company, St. Louis, MO) was added to the incubation mixture. Incubations were terminated by the addition of 1 ml of 1M citric acid. Each sample was incubated with its own control. In the case of the controls, 1 ml of the citric acid was added to the buffer media followed by the substrate (arachidonic acid) and tissue, then it was incubated. The mixtures (control and sample) were extracted twice using the Bligh and Dyer procedure (12). The chloroform layers, containing all the lipids and prostaglandins, were combined and evaporated to dryness under a stream of nitrogen. The residue was purified on a Sephadex LH20 column following the technique described by Jouvenaz et al. (13). Sometimes, further purification by thin layer chromatography was necessary using ethyl acetate-acetic acid-isoctane-water (110:20:50:100, v/v), as developing solvent (14). Recovery of prostaglandin was found to be greater than 95%.

After purification, cholanic acid (Sigma

TABLE III

Endogenous PGE_2 (μg/g) Levels of the Skin of Rats Fed Standard Rat Diet (Chow) or a Semisynthetic Diet Containing One of Several Fat Sources

Diets[a]	Rat number 1	2	3	4	Overall mean[b,c]	Pooled SEM[d]
Chow	68[e]	67	78	81	74[yz]	2.68
Corn	190	153	139	149	158[v]	4.32
Lard	53	50	68	70	60[z]	2.56
L + EA	52	50	57	55	54[z]	2.09
RPF	89	87	104	68	86[xy]	2.43
Zephyr	103	99	96	104	100[w]	1.74
Span	105	91	96	92	96[wx]	2.52
HEAR	105	97	107	109	105[w]	2.61
			Pooled SEM[f]		5.58	

[a]See footnote b, Table I.

[b]Overall mean calculated using 16 observations (4 rats x 4 determinations per rat).

[c]Means with a similar superscript are not significantly ($P < 0.01$) different.

[d]Pooled SEM = pooled standard error of the mean within rat based on the variance between determinations within rat.

[e]Data expressed in terms of micrograms of prostaglandin E_2 per gram of tissue (skin). Each value represents the mean of 4 determinations per rat. Since the variance among determinations and among rats were similar, only the pooled estimates of error for each are given.

[f]Pooled SEM = pooled standard error of the mean, based on the variance between rats within diet.

Chemical Company) was added as internal standard and the combined solution was evaporated to dryness. Prostaglandin methyl esters were prepared by reacting the residue with diazomethane in either for 15 min. Excess reagent and ether were removed under a stream of nitrogen at room temperature. The prostaglandins were further derivatized by reacting the residue with 2% methoxamine in pyridine and then with trimethylsilyl trifluoracetamide and 1% trimethylchlorosilane (BSTFA and TMCS) following the procedure of Horodniak et al. (15).

The derivatized prostaglandins were analyzed by gas chromatography with cholanic acid as internal standard, and using a Hewlett-Packard Model 5710 gas chromatograph equipped with an all glass system (injector to detector) and flame ionization detectors. The following conditions applied: column: 3% SE-30 Ultraphase on 80/100 mesh Chromosorb G, Higher Performance, 180 cm long x 2 mm inside diameter; column temperature: 235 C; flow: 20 ml/min nitrogen; injector port and detector: 250 C. Peaks were quantitated by the use of triangulation procedure and identified by comparison with authentic prostaglandin standards (Sigma Chemical Company). Analyses of variance were determined for all endogenous PGE_2 levels and exogenous synthesis data (16). Differences among treatment means were tested statistically using the Duncan's New Multiple Range Test (16). Since the variance among determinations per rat and between rats across diets were similar, only the pooled estimates of error are given. Correlation between certain dietary constituents and the endogenous PGE_2 levels in skin and exogenous synthesis of this prostaglandin by skin were determined.

RESULTS AND DISCUSSION

The fatty acid composition of the diets fed is shown in Table I. With few exceptions, the diets containing rapeseed oil were lower in saturates and higher in monounsaturates (18:1, 20:1, and 22:1) than were the other diets. The chow (low fat control) and corn oil diets had by far the highest level of linoleic (18:2n-6) acid, the precursor of arachidonic acid which is itself the precursor of PGE_2 (17). The erucic (22:1n-9) acid content of the diets containing rapeseed oil ranged from 0.6 to 23.6 mole %. The RPF diet contained 5.6 mole % esterified erucic acid whereas the L + EA diet contained 5.4 mole % free erucic acid. The 22:1 present in the chow diet probably arises from the presence of fish-meal as part of formulation of this diet and is therefore mainly cetoleic (22:1n-11) acid (18).

The fatty acid composition of the skin used in this study is given in Table II. With but few exceptions, the fatty acid content of the skin reflected that of the diet fed. As expected, the highest content of 18:2n-6 was found in the skin of rats fed the diet containing corn oil, while the highest level of 18:1n-9 was found in

TABLE IV

Correlation of Endogenous Levels of PGE_2 in Rat Skin and Exogenous Synthesis of this Prostaglandin by Skin to Dietary Constituents

Dietary constituent	Values of r for[a]	
	Endogenous levels	Exogenous synthesis
Total monoenes	−0.16	−0.61**
18:1n-9	−0.12	−0.55**
22:1	0.02	−0.10
Total polyunsaturates	0.79**	0.78**
18:2n-6	0.75**	0.83**
18:3n-3	0.13	−0.45**
Total saturates	0.69**	−0.16

[a]**Denotes significance ($P < 0.01$).

TABLE V

Exogenous Synthesis of Prostaglandin E_2 ($\mu g/g/20$ min) by the Skin of Rats Fed a Standard Diet (Chow) or a Semisynthetic Diet Containing One of Several Fat Sources

Diets	Rat number				Overall mean[b,c]	Pooled SEM[d]
	1	2	3	4		
Chow	25.6[e]	18.6	15.9	22.2	20.6^{xy}	1.49
Corn	64.7	58.2	75.0	64.1	65.6^{w}	1.67
Lard	25.3	23.5	19.8	26.2	23.7^{x}	1.09
L + EA	3.5	5.7	2.3	4.8	4.1^{z}	0.60
RPF	8.9	8.3	4.4	6.9	7.1^{yz}	0.85
Zephyr	5.2	3.7	1.7	2.6	3.3^{z}	0.74
Span	11.2	17.5	15.9	14.4	14.7^{y}	0.86
HEAR	21.7	27.8	33.2	14.2	24.2^{x}	1.08
				Pooled SEM[f]	2.21	

[a]See footnote b, Table I.

[b]See footnote b, Table III.

[c]Means with a similar superscript are not significantly different (w,x, $P < 0.01$), (y,z, $P < 0.05$).

[d]See footnote d, Table III.

[e]See footnote e, Table III.

[f]See footnote f, Table III.

the skin of those fed a diet containing either one of the rapeseed oils. Of interest, however, was the relatively low level of 20:4n-6 in the skin of all rats regardless of diet compared to the level of this fatty acid found in heart tissue (8).

The endogenous levels of PGE_2 found in the skin of rats fed these diets are presented in Table III. The highest PGE_2 level observed was in the skin of rats fed the diet containing corn oil. Significantly lower PGE_2 levels were observed in the skin of rats fed all other diets. This is probably not surprising since the corn oil diet contained mush more 18:2n-6, the precursor of 20:4 required for PGE_2 synthesis, than the other diets. The significant ($P < 0.01$) high correlation (Table IV) between the amount of this fatty acid in the diet and endogenous PGE_2 level confirms this observation. The endogenous PGE_2 levels in skin were similar for all rapeseed oil fed grousp (96-105). On the other hand, the PGE_2 levels were significantly higher in rapeseed oil fed rats than in rats fed chow, lard, L + EA, or RPF. The addition of 5.4% erucic acid to lard had no significant effect on endogenous PGE_2 levels, nor did there appear to be a relationship between the level of this fatty acid in the diet and endogenous PGE_2 levels (Table IV).

The levels of PGE_2 in rat skin observed here are considerably higher than those reported earlier (13), possibly due to differences in quantitative recovery. Jouvenaz et al. (19) employing wet homogenization technique, reported endogenous levels of PGE_2 of 100 ng/g for skin and 160 ng/g for vesicular glands of rats. More recently, Jouvenaz et al. (13) using the same homogenization procedure with

stated recoveries of 20-40%, reported levels of 350 ng/g for skin. On the other hand, Tan and Privett (10) reported that it was virtually impossible to obtain quantitative data on the concentration of prostaglandin in animal tissues by wet homogenization and overcame the problem by grinding the tissue in the frozen state. Using the latter technique, they reported levels of PGE_2 in vesicular glands of rats to be from 200 to 850 μg/g, some 1500-fold greater than that reported when wet homogenization is employed (13,19). In the current study, the ground powder technique was employed since attempts to homogenize the tissue in the incubation buffer prior to incubation resulted in yields of less than 5% of that observed when ground powder technique was used, confirming the results of Tan and Privett (10). Thus, the higher endogenous levels of PGE_2 observed in this study compared to those reported earlier (13,19) are probably due to differences in the extraction and determination of these compounds between laboratories, the result of a lack of a universal procedure. Until such a procedure is developed and employed, discrepancies in absolute amounts of these substances in tissues will most likely continue to vary among investigators.

The capacity of the skin of these rats to produce PGE_2 (exogenous synthesis) from arachidonic acid (5,8,11,14-eicosatetraenoic acid) is presented in Table V. The skin of rats fed the corn oil diet demonstrated the greatest capacity to synthesize PGE_2. As indicated in Table IV, there was a significant ($P < 0.01$) negative correlation between the monoene content of the diet and the capacity of skin to synthesize prostaglandin. Skin of rats fed the diet containing corn oil, which had the lowest level of monoenes, had a significantly greater capacity to synthesize PGE_2 than did the skin of all other groups. Conversely, rats fed diets containing rapeseed oil high in monoenes demonstrated the lowest capacity for PGE_2 synthesis. On the other hand, no clear relationship was observed between the level of 22:1 in the diet and the capacity of rat skin to synthesize PGE_2 (Table IV). For example, the skin of rats fed Zephyr rapeseed oil, which contained the lowest level of 22:1, had the lowest capacity for PGE_2 synthesis, whereas the skin of rats fed HEAR oil (23% 22:1) had as high a capacity for PGE_2 synthesis as chow or lard fed rats.

The dermal lesions and alopecia reported earlier (1) are most severe when Zephyr rapeseed oil is fed and much less severe when either Span or HEAR oil is fed. Interestingly, the skin of rats fed Zephyr rapeseed oil demonstrated the lowest capacity for prostaglandin biosynthesis (Table V). Ziboh et al. (20) reported 18:1n-9 to be a strong inhibitor of PGE_2 synthesis from aracidononic acid. In the present study, a significant ($P < 0.01$) negative correlation was found between the level of 18:1n-9 in the diet and the capacity of skin to synthesize PGE_2 (Table IV) which tends to confirm the observation of Ziboh et al. (20).

The relatively high PGE_2 levels found in the skin of rats fed rapeseed oil compared to those fed all other oils but corn oil, and the significantly lower capacity of the skin of these rats to synthesize this prostaglandin, suggests that the turnover of PGE_2 may in fact be affected by feeding rapeseed oil. It has been shown that one of the metabolic pathways for prostaglandin metabolism is by way of β-oxidation and that the process is carnitine dependent (21). It has also been shown that erucic acid or erucylcarnitine has an inhibitory effect on β-oxidation of other fatty acids (22,23). Possibly, inhibition of β-oxidation of PGE_2 by long-chain monoenes may in part account for the relatively high endogenous levels in the skin of rats fed rapeseed oils (67-71% monoenes) compared to when either lard, L + EA, or RPF (47-55% monoenes) was fed.

From the results presented here, it would appear that the dermal lesions and alopecia observed in rats fed diets containing rapeseed oil, could be due to either: (a) the reduced capacity of the skin of these animals to synthesize PGE_2; (b) the reduced rate of turnover of this prostaglandin in rat skin, or; (c) changes in the permeability and functional integrity of the skin due to altered fatty acid composition of this tissue when rapeseed oils are fed. Such a possibility has already been suggested with respect to changes in the functional integrity of other biological membranes (24).

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Alchornea cordifolia Seed Oil: A Rich Source of a New C_{20} Epoxide, (+)*cis*-14,15-epoxy-*cis*-11-eicosenoic Acid

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ABSTRACT

A C_{20} homolog of vernolic acid has been found at the 50% level in *Alchornea cordifolia*, Euphorbiaceae, seed oil. This new acid, (+)*cis*-14,15-epoxy-*cis*-11-eicosenoic (alchornoic) acid, was isolated by high-pressure liquid chromotography and characterized by mass spectrometry, nuclear magnetic resonance and infrared spectroscopy, optical rotary dispersion, and ozonolysis-gas-chromotography.

INTRODUCTION

Epoxy fatty acids have long been known to be seed oil constituents (1,2). Those found have all been 18-carbon acids with the epoxy group located in one of three positions: Δ9, Δ12, or Δ15; two stereoisomers are known for both the Δ9 and Δ12 positions. We now report a new epoxy acid, (+)*cis*-14,15-epoxy-*cis*-11-eicosenoic (alchornoic) acid, which makes up over 50% of the acyl groups of the seed oil from *Alchornea cordifolia* (Schum. & Thonn.) Muell.-Arg., a Euphorbiaceous plant collected in Ghana.

EXPERIMENTAL PROCEDURES

Oil, 46% (dry basis), was extracted from the ground seed with petroleum ether using a Butt extractor. Oxirane oxygen determination with HBr, infrared (IR), nuclear magnetic resonance (NMR), and gas chromatography-mass spectrometry (GC-MS) analyses were accomplished as previously described (3-5).

Thin layer chromatography (TLC) of the seed oil was performed on plates spread with 250 μ layers of Silica Gel G. Hexane-diethyl ether (70:30) was the developing solvent. The oil was tested for epoxy groups on the TLC plate using the picric acid method of Fioriti and Sims (6).

Methyl esters were prepared from the oil by two procedures: (a) reaction with diazomethane, followed by transesterification with 0.1% sodium methoxide in methanol, and (b) saponification in 0.5 N NaOH in methanol followed by reaction with 10% BF_3 in methanol.

A Hewlett-Packard model 402 gas chromatograph was used for methyl ester analyses. The chromatograph was equipped with a 10-ft x 4-mm ID glass column packed with 5% LAC-2-R 446 and a 4-ft x 4-mm ID glass column packed with 5% Apiezon L. Analyses were made isothermally at 185 C.

Epoxy esters were separated from the unoxygenated ones on 25 g of 60/200 mesh Hi-Flosil)Applied Science Laboratories, Inc., State College, PA) packed in a 15 mm ID column. The esters were eluted with hexane containing increasing amounts of diethyl ether. Progress of the separation was monitored by TLC. The two components of the epoxy ester fraction were separated with a Waters model ALC-201 liquid chromatograph equipped with a 30-cm x 1/4-in. C_{18}-μBondapack column. The elution solvent was acetonitrile-water (80:20) at 2 ml/min.

The epoxy esters were ozonized as previously described (7), and the ozonides were reduced directly in the gas chromatographic column. To accomplish this, a 6-ft x 2-mm glass column was packed with 5% Apiezon L. except for the first 6 in. which were filled with 1% palladium on 42/60 mesh Chromosorb P (8). With hydrogen as the carrier gas, the ozonides were catalytically reduced and the products were subsequently separated by the column and then directed to a mass spectrometer.

RESULTS AND DISCUSSION

Large amounts of epoxy groups were indicated in *A. cordifolia* oil by its hydrogen bromide uptake (57%, calculated as epoxyoleic acid) and moderate IR absorption at 827 and 848 cm^{-1}. TLC of the oil showed components with the same migration characteristics as free vernolic acid, monovernoyl, divernoyl, and trivernoyl triglycerides, when compared to *Vernonia anthelmintica* oil. These components gave an orange color when reacted with picric acid, characteristic of epoxy groups (6).

GC analysis of the base catalyzed esters (Table I) revealed two unusual components. The smaller of the two (2.3%) had equivalent chain lengths (ECLs) of 19.0 from the Apiezon L column and 22.9 from the LAC-2-R 446 column, identical to authentic methyl vernolate. The major fatty ester had ECLs of 21.1 (Apiezon L) and 25.2 (LAC-2-R 446). These ECLs are ca. 2 units greater than those of methyl vernolate and indicated a similar fatty ester with two more methylene units. The mass

TABLE I

Fatty Acid Composition of *Alchornea cordifolia* Seed oil, % by Gas Liquid Chromatography

14:1	0.1	18:0	1.2	20:0	0.1
15:0	0.2	18:1	15.7	20:1	0.2
16:0	12.8	18:2	13.2	20:2	0.1
17:1	0.1	18:3	0.8	unknown	1.7%

Vernolic acid 2.3

Alchornoic acid 51.2

spectrum of the smaller component was indistinguishable from authentic methyl vernolate (MW = 310), and the major component exhibited a molecular ion of m/e 338. These spectra were not definite enough to locate the epoxy groups (5), and so methoxy-hydroxy derivatives were formed from the epoxy acyl groups by BF_3-methanol treatment (9). After silylation, these compounds give spectra which can be used to locate the orignal epoxy function (5) as illustrated in Figure 1. Here, the spectrum of derivatized methyl alchornoate and the structures of the ions, which locate the methoxy and trimethylsilyloxy groups, are shown. These intense ions locate the epoxy group in the 14,15 position. In addition, the position of the double bond at $\Delta 11$ is indicated by the abundance of ions with m/e 217. In silylated derivatives of diols or methoxy-hydroxy esters, the primary cleavage is between oxygenated carbon atoms unless there is a double bond located one methylene unit from the oxygenated carbons in which cleavage between the double bond and the oxygenated carbon becomes important (5). If the double bond was conjugated to the epoxy group or separated by more than one methylene group, the m/e 217 ion would be much less intense or not found at all.

The location of the olefinic group was substantiated by both ozonolysis and NMR. Ozonolysis of the intact epoxy-ester fraction produced three major fragments: C_9 epoxy aldehyde, C_{11} aldehyde-ester, and C_{10} methyl ester (thermal degradation of the C_{11} aldehyde-ester). In addition to these major fragments, minor fragments (C_9 epoxy aldehyde, C_9 aldehyde-ester, and C_8 methyl ester) were observed from methyl vernolate.

No absorption for *trans* olefins was detected in the IR spectrum (960 cm^{-1}) and, therefore, the double bonds are in the *cis* configuration.

NMR data also were consistent with the epoxy monoenoic structure for the major acyl group from *A. cordifolia.* Proton signals were observed at 0.9δ (terminal methyl), 1.28-1.5δ (chain methylenes), 2.03δ (methylene adjacent to both the epoxy and olefinic groups), 2.29δ(c-2 protons), 2.90δ(*cis*-epoxy), 3.62δ (methoxyl), and 5.42δ (olefinic). The location of the methylene generating the signal at 2.03δ was established, since both the epoxy and olefinic proton signals collapsed when irradiated at 2.03δ.

Optical rotary dispersion (ORD) of the pure *cis*-14,15-epoxy,*cis*-11-eicosenoate, isolated by high-pressure liquid chromatography (HPLC), showed specific rotations of $[\alpha]_D^{26}$ + 2.24, $[\alpha]_{560}$ + 2.43, $[\alpha]_{520}$ + 2.66, $[\alpha]_{480}$ + 3.07, $[\alpha]_{400}$ + 4.21, $[\alpha]_{360}$ + 4.51, $[\alpha]_{335}$ + 4.62, $[\alpha]_{320}$ + 4.35, $[\alpha]_{300}$ + 3.40, $[\alpha]_{280}$ + 1.52 (C. 2.38). The ORD spectrum has the same sign and shape as that of methyl vernolate (10). Therefore, the configuration of methyl alchor-

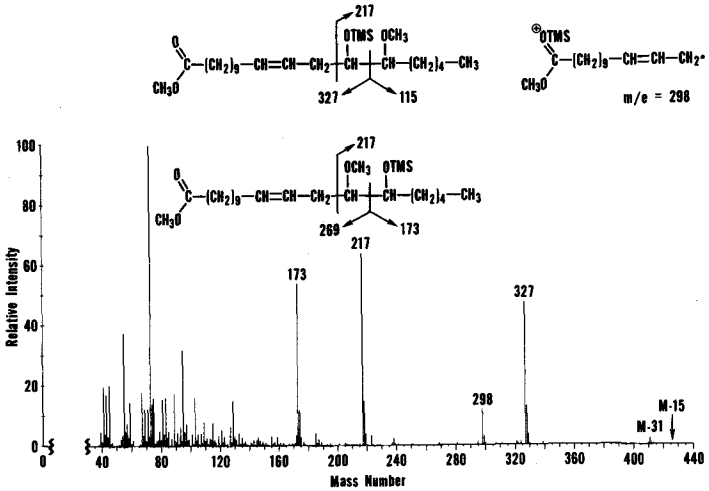

FIG. 1. Mass spectrum of the silylated BF_3-methanol derivative of methyl alchornoate.

noate (14*S*,15*R*) is most likely the same as (+) methyl vernolate (12*S*,13*R*) (10).

Three other Euphorbiaceae species, *Euphorbia lagascae* (11), *Cephalocroton cordofanus* (12), and *Cephalocroton pueschelii* (13), have been reported to produce seed oils containing large amounts of vernolic acid. We speculated that, in the past, minor amounts of alchornoic acid could have been overlooked in the analysis of oils from these species. However, GC experiments designed to find alchornoic acid at levels less than 0.1% failed to detect methyl alchornoate in oils from these species. It appears that the biosynthetic production of alchornoic acid either results from epoxidation of a C_{18} acid and subsequent chain elongation, as indicated from the small amount of vernolic acid present, or from a C_{20} acid directly.

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Oxygenated Fatty Acid Constituents of Soybean Phosphatidylcholines[1]

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ABSTRACT

Bitter-tasting phosphatidylcholines from hexane-defatted soybean flakes were chromatographically separable from ordinary soy phosphatidylcholines (SPC). The bitter-tasting SPC contain 32% oxygenated fatty acids in addition to palmitic, stearic, oleic, linoleic, and linolenic acids. Identification of these oxygenated acids was based on infrared, ultraviolet, proton nuclear magnetic resonance, and mass spectral characteristics of methyl ester derivatives which were separated and purified by column and thin layer chromatography. The fatty acid methyl esters identified were (a) 15,16-epoxy-9,12-octadecadienoate, (b) 12,13-epoxy-9-octadecenoate, both with double bonds and epoxide groups predominantly of *cis* configuration; (c) 13-oxo-9,11- and 9-oxo-10,12-octadecadienoates; (d) 13-hydroxy-9,11- and 9-hydroxy-10,12-octadecadienoates; (e) 9,10,13-trihydroxy-11- and 9,12,13-trihydroxy-10-octadecenoates. In addition, trace amounts of (f) 11-hydroxy-9,10-epoxy-12- and 11-hydroxy-12,13-epoxy-9-octadecenoates; (g) 13-oxo-9-hydroxy-10- and 9-oxo-13-hydroxy-11-octadecenoates; (h) 9,10-dihydroxy-12- and 12,13-dihydroxy-9-octadecenoates; and (i) 9,12,13-dihydroxyethoxy-10- and 9,10,13-dihydroxyethoxy-11-octadecenoates were indicated by mass spectrometry. Dihydroxyethoxy compounds (i) were possibly formed upon extraction of the SPC from flakes by 80% ethanol. Except for the first two epoxy compounds, labelled a and b, the oxygenated fatty acids are similar to the products formed by homolytic decomposition of linoleic acid hydroperoxide. The first two compounds with predominantly *cis* configuration may occur by action of fatty acid hydroperoxides on an unsaturated fatty acid.

INTRODUCTION

Seed oils containing oxygenated fatty acids occur in a wide variety of plants including soybeans (1-3). These fatty acids increase during seed storage (3-5) and may adversely affect flavor and nutritional properties of the seed. Their possible formation has been the subject of numerous studies with model systems of lipoxygenase or hydroperoxide-decomposing enzymes from cereals, peas, and soybeans acting either on the unsaturated fatty acid or its hydroperoxide as the substrate (6-17).

Sen Gupta identified some monoepoxy and hydroxy unsaturated fatty acids contained in the triglycerides and phospholipids in soybean oil (18). Sessa et al. found oxygenated fatty acids in the bitter-tasting soybean phosphatidylcholines (SPC) isolated from defatted flakes (19). To remove SPC bound to the protein after defatting the flakes with n-hexane, hydrogen bond-breaking solvents containing alcohol were used. In this latest investigation, the oxygenated fatty acids of SPC isolated from defatted flakes were identified.

MATERIALS AND METHODS

Isolation, Purification, and Taste Evaluation of SPC

The method used to isolate and purify SPC is the same as previously reported (19) except that the step involving catalytic hydrogenation of phospholipids was eliminated. To minimize oxidative deterioration of the phospholipids during their isolation, chromatography was performed under nitrogen atmosphere. Solvents from column and thin layer chromatography (TLC) streak preparation fractions were stripped to near dryness on a rotary evaporator, below 25 C. Fractions were redissolved in an azeotropic mixture of n-hexane-ethanol (82:18 v/v) as dilute solutions and stored in the dark under nitrogen atmosphere at −18 C until needed.

In a previous study by Sessa et al. (19), SPC were separated into three fractions, SPC-A, -B, and lyso-SPC. Portions of the fractions were evaluated for intensity of bitter taste by a formal taste panel. The isolated bitter-tasting SPC remaining after taste panel evaluation were consolidated and stripped of solvent to yield 285 mg of starting material.

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Chromatography

Fatty acid methyl esters (FAME) extracted from SPC after transesterification with sodium methoxide, in 50-mg portions, were separated by column chromatography on 10 g acid-treated Florisil (Supelcosil ATF-061, Supelco Inc., Bellefonte, PA). The column (1.0 X 18.6 cm) was eluted successively with: 80 ml 1% diethyl ether in n-hexane followed by 80 ml 10% ether, 100 ml 25% ether, and 100 ml 50% ether; then 50 ml 5% methanol in ether, 100 ml 25% methanol, and 100 ml 50% methanol at a flow rate 150 ml/hr. Effluents, ∿14 ml/tube, were monitored at 232 and 274 nm and also by TLC on 0.25 mm precoated Silica Gel F-254 plates (E. Merck, Darmstadt, Germany, distributed by Brinkman Instruments, Inc., Waterbury, NY) developed with n-hexane-diethyl ether, 1:1 v/v. Spots were visualized by spraying with 0.5% potassium dichromate in 50% sulfuric acid followed by heating 30 min at 150 C. Effluents either comprising an ultraviolet (UV) absorbing peak or showing similar content by TLC analysis were pooled. Resulting column fractions found to be mixtures were purified further by TLC on preparative plates (20). For most of the FAME, the solvent system n-hexane-diethyl ether, 1:1, gave adequate separation; more polar components with R_f below 0.5 required hexane-ether-acetic acid, 50:50:1, for their purifications; those fractions containing multiple hydroxyl groups were silylated (see Derivatives section) and then purified on short columns of acid-treated Florisil packed in n-hexane and eluted with hexane-ether (dried with anhydrous sodium sulfate) mixtures.

The purified samples were rechromatographed on TLC to analyze for homogeneity and to characterize by spray reagents. FAME were detected with phosphomolybdic acid spray; spots containing epoxy compounds were located with picric acid (21) and carbonyl compounds with 0.4% 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid.

Composition of unoxygenated FAME was determined by gas liquid chromatography (GLC) on a 6 ft X 0.4 cm (ID) column packed with 20% DEGS on Gas Chrom P, carrier gas-helium 40 ml/min, oven isothermal 180 C, inlet temperature 220 C, detector 215 C, and was recorded as percentage of total gas liquid chromatographic peak area. FAME containing epoxy, oxo, or silylated hydroxyl groups were separated by GLC on either 3% Silar 5CP on Gas Chrom Q or 3% OV-1 on Gas Chrom Q with conditions described by Gardner et al. (22).

Derivatives

Methoxy, hydroxyl derivatives of epoxy FAME are formed by reaction of the epoxide with BF_3/methanol (23). Silylation of these derivatives produces compounds which upon mass spectroscopy give simplified spectra that define the location of the original epoxy ring (24).

Oxo groups were reduced with excess sodium borohydride in methanol (25); unsaturated FAME were hydrogenated in methanol with a 10% palladium on carbon catalyst at 1 atm and 25 C.

Silyl derivatives of monohydroxylated and multihydroxylated FAME were prepared according to Gardner et al. (22).

Spectroscopy

Infrared (IR) spectra were determined as described previously (22).

High resolution nuclear magnetic resonance (NMR) spectra were taken in deuteriochloroform with either a Varian Model HA-100 spectrometer at 100 MHz or a Bruker WH-90 operating at 90 MHz with 1% tetramethylsilane used as an internal standard. When tetramethylsilyl (TMS) ether derivatives were analyzed, only a trace amount of tetramethylsilane was used as a reference standard.

Mass spectrometry (MS) was employed in tandem with GLC as described by Kleiman and Spencer (24). Multiple mass spectra were recorded throughout the GLC elution of each methyl ester to detect positional isomers.

RESULTS

Separation of FAME

The column fractions, labelled A through G, when chromatographed on TLC, showed a complex array of spots with different polarities. Recovered weights of crude fractions A through G were 129, 18, 5, 20, 8, 3, and 7 mg, respectively. Purification of these by preparative TLC led to either partial or complete removal of those spots designated by dotted lines in Figure 1.

Unoxygenated FAME

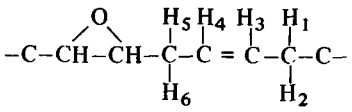
Fraction A contained a mixture of unoxygenated FAME, which, when analyzed by GLC, had the following composition: 22.2% 16:0, 5.6% 18:0, 19.6% 18:1, 50.1% 18:2, and 2.4% 18:3.

Epoxy FAME

Fraction B (99% pure by GLC) gave a positive color reaction for epoxy compound with

picric acid on TLC analysis and had a GLC retention time identical to methyl vernolate, a 12,13-epoxy-9-octadecenoate. It had little or no absorbance in UV, thus indicating no conjugated double bonds. Its IR spectrum in carbon disulfide suggested an isolated *cis* double bond with absorption at 3010 and 720 cm^{-1} (11), a carbonyl of a methyl ester at 1740 and 1170 cm^{-1}, and *cis*-epoxide with moderately intense bands at 820 and 840 cm^{-1} (26). Very weak absorptions at 960 cm^{-1}, *trans* monoene, and at 885 cm^{-1}, *trans*-epoxide, indicated little contribution of *trans* isomers.

NMR spectrum of B has the following absorptions with proton integration, signal multiplicity, and probable assignments in parentheses: δ0.9 (3H; t; $C\underline{H}_3$-C–); δ1.31 (20H; broad s; $-C\underline{H}_2-$); δ2.01 (2H; d; $-\underset{O}{\overset{/\backslash}{C}}-\underset{}{\overset{}{C}}-C=C-C\underline{H}_2-$); δ2.29; (4H; t; $-C\underline{H}_2-COO-$ and $-C=C-C\underline{H}_2-\underset{O}{\overset{/\backslash}{C}}-C-$); δ2.91 (2H; m; cis $-\underset{O}{\overset{/\backslash}{H\underline{C}-C\underline{H}}}-$) (27); δ3.64 (3H; s; $COOC\underline{H}_3$); δ5.45 (2H; m; –HC=CH–). A partially obscured triplet at δ1.05 is indicative of some linolenate (ca. 18%) in this sample. Double irradiation experiments indicated the allylic methylene, $-C=C-C\underline{H}_2-\underset{O}{\overset{/\backslash}{C}}-C-$, which is obscured by the unsymmetrical triplet at δ2.29. In addition, the chemical shifts and coupling constants for the structure



were δ5.35 = H_4; δ5.53 = H_3; $J_{3,4}$ = 10 Hz; $J_{1,3} = J_{2,3}$ = 5 Hz; $J_{4,5} = J_{4,6}$ = 6 Hz, and are consistent with those expected from a first order analysis of a *cis*-olefin. Double irradiation of the epoxide proton β to the olefin at δ2.91 had no effect on the olefinic absorption, thus showing the epoxide was not adjacent to the unsaturation.

Because mass spectra of unsaturated epoxy FAME are difficult to interpret, methoxy-hydroxy derivatives are formed by cleaving the epoxide with BF_3/methanol (23). Although two products are formed for each epoxide, GLC analysis is little affected, since with non-polar columns such as Silar SCP and OV-1 both products emerge together. TMS ether derivatives of the methoxy-hydroxy compounds derived from fraction B showed two major peaks using both columns which indicate two

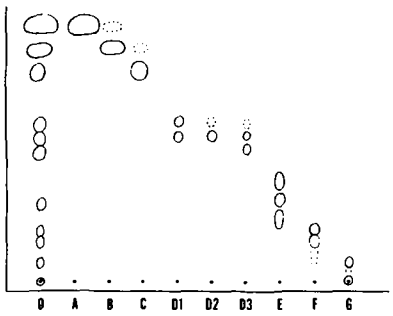
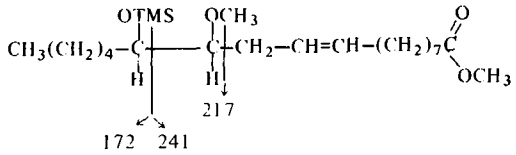
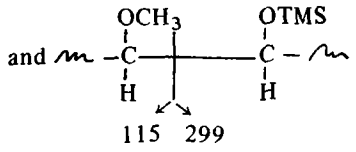


FIG. 1. Thin layer chromatography tracing of fatty acid methyl ester mixture from transesterified soy phosphatidylcholines (O) and column fractions A through G on silica gel plates (0.25 mm thickness) developed with n-hexane-diethyl ether, 1:1 v/v. Spots with dotted lines represent material removed by streak prep., thin layer purification of the column fractions.

different positional isomers. GLC-MS of the first peak gave a spectrum identical to the TMS derivative of 12,13-epoxy-9-octadecenoate $[CH_3(CH_2)_4\underset{O}{\overset{/\backslash}{CH-CH}}-CH_2CH=CH(CH_2)_7COOCH_3]$ after reaction with BF_3/methanol (24). Its mass spectrum shows a molecular ion (M) at m/e 414, 399 (M – 15), and 383 (M – 31). Other characteristic peaks noted were m/e 299, 270, 241, 217, 173, and 115 which corresponded with the fragments represented in the formulae below.



and



GLC-MS of the second peak (illustrated in Fig. 2) showed a molecular ion at m/e 412, 381 (M – 31), a rearrangement ion at m/e 310 and peaks at m/e 339, 175, and 131 characteristic of the fragments represented by formulae given in the figure. 15,16-Epoxy-9,12-octadecadienoic acid $[CH_3CH_2\underset{O}{\overset{/\backslash}{CH-CH}}-CH_2CH=CHCH_2CH=CH(CH_2)_7COOCH_3]$, rarely found in seed oils, is probably derived from linolenic acid. The more common 9,10-epoxy-12-octadecenoate

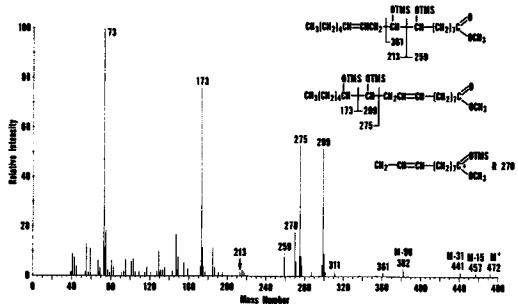


FIG. 2. Mass spectrum of an epoxy fatty acid methyl ester from Fraction B after reaction with BF_3/methanol and trimethylsilylation with bis(trimethylsilyl)-trifluoroacetamide.

$[CH_3(CH_2)_4CH{=}CHCH_2\underset{\diagdown\,\diagup}{\overset{O}{CH}}{-}CH(CH_2)_7COOCH_3]$ reportedly present in soybean oil triglycerides and phospholipids (18) was not evident in fraction B.

Oxodiene FAME

Crude fraction C contained two spots when analyzed by TLC and two major peaks by GLC. The less polar component possessed the chromatographic mobility, GLC retention time, and MS of the 12,13-epoxy compound isolated and identified in fraction B. The more polar component, with lesser chromatographic mobility on TLC, gave a positive color reaction with 2,4-dinitrophenylhydrazine spray reagent indicative of a carbonyl group. This compound, purified by TLC (see Fig. 1), possessed a single sharp maximum at 267 nm in cyclohexane and at 275 nm in methanol with an ϵ max of ca. 20,000. The shift in UV absorbance in cyclohexane versus methanol solvents is typical of conjugated oxodienes (28). IR spectrum of a solution in carbon tetrachloride had bands at 1695, 1685, 1640, and 1600 cm^{-1} all associated with the oxodiene system (17,28), 990 and 960 cm^{-1} for *cis-trans* conjugated double bond, and a 1740 cm^{-1} band resulting from ester carbonyl stretching.

Characteristic NMR peaks of fraction C and their respective assignments are: δ0.89 (t; CH_3-C–); δ1.31 (broad s; $-CH_2$); δ1.63 (s; $CH_2-C-C{=}C-$); δ2.23 (d; $CH_2-C{=}C$); δ2.31 (t, CH_2COO-); δ2.54 (t; $CH_2\underset{\|}{C}-C{=}C-$); δ3.67 (s; CH_3OCO-); δ6.06 (dd; $C{=}CH-\underset{\|}{C}-$); δ6.21 (m; $-CH{=}CH-$); δ7.02 (dd; $CH{=}C-\underset{\|}{C}-$).

Mass spectrum of fraction C gave peaks comparable to those reported by Arens and Grosch (6) for a methyl ester mixture of the 9-oxo-10,12- $[CH_3(CH_2)_4CH{=}CH-CH{=}CH\overset{O}{\overset{\|}{C}}(CH_2)_7COOCH_3]$ and 13-oxo-9,11-octadecadienoic acids $[CH_3(CH_2)_4\overset{O}{\overset{\|}{C}}CH{=}CH-CH{=}CH(CH_2)_7COOCH_3]$.

Catalytic hydrogenation of fraction C yielded a mixture of tetrahydro derivatives the mass spectra of which were identical with 13- and 9-oxooctadecanoates reported by Ryhage and Stenhagen (29).

Reduction of the keto group of these oxooctadecanoates with sodium borohydride followed by trimethyl silylation yielded derivatives whose mass spectra confirmed the position of the oxygen functions at the 9- and 13-positions (13).

Hydroxydiene FAME

Preparative TLC of column fraction D separated two spots from the third, thereby yielding three subfractions designated D1, 2, 3 (see Fig. 1). UV spectra of these subfractions showed strong maxima at 234, 233, and 231, respectively, all of which indicate presence of a conjugated dienol. All three gave IR spectral absorptions at 1730 and 1173 cm^{-1} for carbonyl of methyl ester and 3620 and 3500 cm^{-1} (broad) for a hydroxyl group. D1 and D2 showed *cis-trans* conjugated double bonds with absorbing bands at 3010, 985, and 950 cm^{-1} (6) whereas D_3 the lower spot, showed increased ratio of 985 to 950 cm^{-1} indicative of increased admixture of *trans,trans* with *cis,trans* (25). Based on chromatographic and spectral examinations and findings from previous work (25), the components of these subfractions were presumed to be the 13-*cis,trans;* 13-*trans, trans;* 9-*cis,trans*; and 9-*trans,trans* isomers with the second and third isomers overlapping on TLC analysis.

Based on NMR spectrum D1 is mostly the 13-hydroxy-*cis*-9, *trans*-11-octadecadienoate $[CH_3(CH_2)_4\underset{|}{\overset{OH}{CH}}{-}CH{=}CH{-}CH{=}CH(CH_2)_7COOCH_3]$ isomer. Its spectrum is similar to the one reported by Tallent et al. (30) except for slight downfield absorptions of the protons on carbons-9 (δ5.43 vs. 5.30), -10 (δ6.00 vs. 5.90), -11 (δ6.50 vs. 6.35), -12 (δ5.67 vs. 5.56), and -13 (δ4.13 vs. 4.05). The coupling constants were in agreement with those reported in the literature (30,31).

D1 through 3 were pooled, silylated, and subjected to GLC-MS analyses. The derivatives gave a molecular ion peak at m/e 382 and peaks

with m/e at 311 and 225 characteristic of fragments produced by TMS ether derivatives of 13- and 9-hydroxy-octadecadienoates (24).

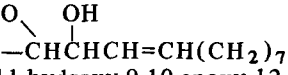
Catalytic reduction of consolidated fraction D yielded products which when silylated and subjected to GLC-MS confirmed the presence of the two isomers with hydroxyl groups at the 9- and 13-positions.

Difunctional Oxidized FAME

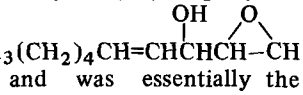
TLC analysis of Fraction E showed one well-defined spot which gave positive color reaction with 2,4-dinitrophenylhydrazine spray reagent and two diffusely charring zones ahead of and behind it. Because of the small quantity of this fraction and its apparent complexity based on GLC of its silyl derivatives, no attempt was made to isolate and purify the components by TLC. UV spectrum showed a broad maximum at 225 nm. IR spectrum in carbon tetrachloride featured characteristic absorptions at 3620 cm^{-1} for hydroxyl group, 3500 cm^{-1} (broad band) for intermolecularly bonded hydroxyl group, 1740 cm^{-1} for ester carbonyl, 1717 cm^{-1} for oxo-carbonyl, and 980 cm^{-1} for isolated *trans* olefin.

The integrated NMR curve of the TMS ether derivative indicated more than one hydroxyl group. Except for a terminal methyl unsymmetrical triplet at δ0.89, a methylene broad peak with top at δ1.30, a methylene adjacent to a carboxylate at δ2.32, and a singlet at δ3.67, the absorptions were poorly defined.

GLC-MS of silylated Fraction E yielded a multicomponent system. One series of mass spectra of a GLC peak appeared to consist of isomers 11-hydroxy-12,13-epoxy-9-



$[CH_3(CH_2)_4CH-CHCHCH=CH(CH_2)_7COOCH_3]$ and 11-hydroxy-9,10-epoxy-12-



octadecenoates $[CH_3(CH_2)_4CH=CHCHCH-CH(CH_2)_7COOCH_3]$ and was essentially the same as reported by Gardner et al. (22). The mass spectra showed the following: a small molecular ion with its intensity at m/e 398 (0.5), 383 (3.3; M – 15), 327 (1.5), 298 (2.8), 285 (53.6), 241 (2.2), 199 (25.5), 155 (22.0), 129 (26.1). Intensities shown above in parentheses are all relative to m/e 73.

Mass spectra of a second GLC peak possessed a molecular ion m/e 398, 383 (M – 15), 367 (M – 31), and fragment ions 327, 259, 241, 237, 199, 185, 173, 99, and 73 indicative of silylated 13-oxo-9-hydroxy-10-$[CH_3(CH_2)_4$

$C-CH_2CH=CHCH(CH_2)_7COOCH_3]$ and 9-oxo-13-hydroxy-11-octadecenoates

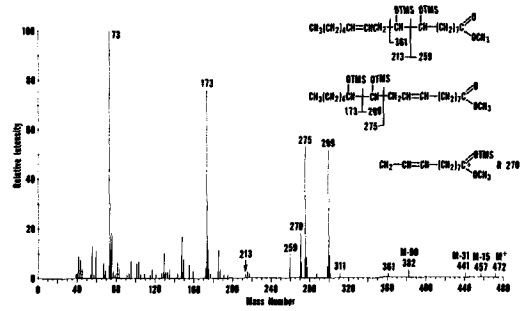
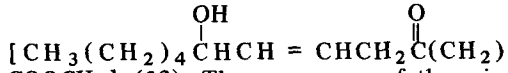
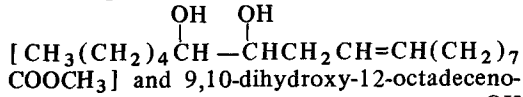


FIG. 3. Mass spectrum of TMS ether derivatives of dihydroxyoctadecenoates from Fraction E.

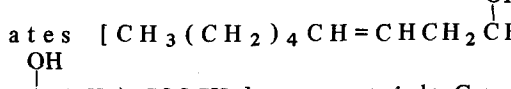


$[CH_3(CH_2)_4CHCH=CHCH_2C(CH_2)_7COOCH_3]$ (22). The oxo group of these isomers undoubtedly gave the positive color reaction when the developed thin layer chromatogram was sprayed with 2,4-dinitrophenylhydrazine reagent.

Mass spectra of a third GLC peak, Figure 3, demonstrated fragment ions characteristic of the two positional isomers 12,13-dihydroxy-9-



$[CH_3(CH_2)_4CH-CHCH_2CH=CH(CH_2)_7COOCH_3]$ and 9,10-dihydroxy-12-octadecenoates



$[CH_3(CH_2)_4CH=CHCH_2CH-CH(CH_2)_7COOCH_3]$ as reported by Graveland et al. (32).

Because of the complexity and insufficient quantities of Fraction E, the positions of the oxygen functions were not confirmed by chemical and physical methods.

Fraction F, representing about 1.6% of the entire fatty acid mixture, gave two spots on TLC analysis, but its TMS ether derivative gave only one peak on GLC with a Silar 5CP column. This fraction showed no absorption maximum in the UV region. IR spectrum in carbon tetrachloride showed hydroxyl absorption at 3500 cm^{-1} (broad), ester carbonyl absorption at 1740 and 1170 cm^{-1}, ether absorption at 1085 cm^{-1}, and isolated *trans* unsaturation at 980 cm^{-1}. NMR of the TMS ether derivative showed a peak at δ0.08 confirming the presence of converted hydroxyl groups. The remaining absorptions, though not as well defined, were similar to those reported for dihydroxyl ethoxyoctadecenoic acid by Gardner et al. (22).

MS of trimethylsilylated Fraction F gave the following fragments with intensitites in parentheses at m/e 416 (1.66), 387 (2.44), 343

(1.36), 297 (3.51), 259 (44.4), 173 (60.8), 155 (9.9), 129 (8.4), 103 (11.7), 83 (6.5), 75 (20.1), 73 (100.0), and except for minor differences in intensities was identical to the spectrum of trimethylsilylated dihydroxyethoxyoctadecenoate isomers reported by Gardner et al. (22).

Trihydroxy FAME

Fraction G exhibited nonspecific absorption over the wavelength region 220-360 nm. Its IR spectrum showed absorptions at 3400 cm^{-1} (broad) indicative of a polyhydroxy compound, 1740 and 1170 cm^{-1} for an ester carbonyl and 975 cm^{-1} for an isolated *trans* double bond. NMR spectrum was identical to that reported by Graveland (13), except for upfield displacement of a singlet from $\delta 3.6$ to $\delta 1.97$. Displacement of hydroxyl protons is not unusual because location of this singlet is dependent on certain physical parameters such as concentration. NMR spectrum of the TMS ether derivative had no $\delta 1.97$ absorption peak but did have a new peak at $\delta 0.1$ which gave integration of 27H equivalent to three hydroxyl groups.

MS of silylated Fraction G was in accordance with that described in the literature (6, 13, 15, 33-35) for a mixture of trimethylsilylated 9,12,13-trihydroxy-10- $[CH_3(CH_2)_4\overset{OH}{\underset{|}{C}}H\overset{OH}{\underset{|}{C}}HCH=\underset{OH}{\underset{|}{C}}HCH(CH_2)_7COOCH_3]$ and 9,10,13-trihydroxy-11-octadecenoates $[CH_3(CH_2)_4\overset{OH}{\underset{|}{C}}HCH=\underset{OH}{\underset{|}{C}}H\overset{OH}{\underset{|}{C}}HCH(CH_2)_7COOCH_3]$.

DISCUSSION

A model system with soybean lipoxygenase and linoleic acid hydroperoxide in the presence of an electron donor was previously reported to produce hydroxy- and oxo-octadecadienoic acids as well as dihydroxy-, hydroxyepoxy-, and trihydroxy-octadecenoic acids (15). In this study, we identified oxygenated fatty acids from an isolated soy phospholipid that were identical to those generated in the model system. In addition, 12,13-epoxy-9-octadecenoic and 15,16-epoxy-9,12-octadecadienoic acids were identified. The epoxy and olefinic groups in these compounds are predominantly in the *cis* configuration and can possibly arise by action of a fatty acid hydroperoxide on unsaturated fatty acid. The formation of the 15,16- and 12,13-epoxides in the absence of the analogous 9,10 epoxy compound suggests a possible enzyme-mediated reaction.

We propose that decomposition of hydroperoxides in legumes differs from that in cereals (36). Virtually all the oxygenated fatty acids derived from soy, regardless of whether isolated from enzymic systems or SPC from defatted flakes, are identical to those oxygenated fatty acids obtained by degrading hydroperoxides in model systems with ionic iron-cysteine or hemoglobin. For example, the isomeric mixture of δ-ketols, 13-oxo-9-hydroxy-10- and 9-oxo-13-hydroxy-11-octadecenoic acids was detected by GLC-MS in one of our soy phospholipid fractions. By comparison, in cereals, hydroperoxide degradation occurs by a linoleic acid hydroperoxide isomerase which gives rise to the α-ketols: 10-oxo-13-hydroxy-11- and 12-oxo-9-hydroxy-10-octadecenoic acid. It could be concluded that the δ-ketols found in soybeans are not formed by an isomerase reaction but by an autoxidation reaction perhaps catalyzed by a metallo protein.

Even though the physiological role of lipoxygenase in plant metabolism is unknown, the possibility exists that homolytic decomposition of the hydroperoxide in polyene phospholipids may drastically alter membranes in the cell by creating less hydrophobic regions in the lipid phase. Also it is not known that role the oxygenated fatty acids have in metabolism.

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Composition of *O*-Alkyl and *O*-Alk-1-enyl Moieties in the Glycerolipids of the Human Adrenal

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ABSTRACT

A comparison of human adult and fetal adrenals with respect to their levels of glyceryl ether lipids and other lipid components is reported. Fetal glands contained significantly lower levels of alk-1-enyl phosphoglycerides and of cholesterol. Neutral glyceryl ether diesters, and ethanolamine and choline phosphoglycerides were isolated from adult adrenal tissue. The composition of the *O*-alkyl glycerol groups in these lipid fractions was obtained by means of gas chromatography of the trimethylsilyl ethers and diacetyl derivatives; *O*-alk-1-enyl glycerols were analyzed as their diacetates. About one-half of the alkyl and alk-1-enyl glycerol moieties present in glyceryl ether diesters contained hydrocarbon side chains with 20, 22, or 24 carbon atoms. Long hydrocarbon chains (C_{19-24}) were also found in the *O*-alkyl glycerol moieties present in the total lipids of fetal adrenals.

INTRODUCTION

Glycerolipids which contain ether bonds are found ubiquitously in mammalian tissues, but little is known about the ether lipids present in the human adrenal (1). The recent finding that alkyl diacylglycerols accumulated in the adrenals of an infant with Wolman's disease drew attention to the fact that neutral glyceryl ether lipids were also present in this gland, besides the plasmalogens (2). This paper concerns six classes of ether lipids found in normal adrenals. Most of the analyses were done on specimens taken from adults. Fetal adrenals (at 28-36 wk of maturity) were also studied because this gland exhibits a specific pattern of development in the prenatal period (3). Neutral lipids and phospholipids containing *O*-alkyl and *O*-alk-1-enyl moieties were found in both fetal and adult tissues. The compositions of the *O*-alkyl and *O*-alk-1-enyl glycerol moieties in the six lipid classes are reported. The scope of the observations on adrenal ether lipids made earlier by Debuch and Winterfeld (4,5) has therefore been extended.

MATERIALS AND METHODS

Chemicals

The following commercial preparations were used: hexadecyl and octadecyl glycerol, glycerol 1,2-diarachidoyl-3-eicosanyl ether (Analabs, Inc., North Haven, CT); tetradecyl glycerol, 1-octadec-9-enyl glycerol, Sil-Prep (Applied Science Laboratories, Inc., State College, PA); phospholipase C from *B. cereus* (Sigma Chemical Co., St. Louis, MO); phosphatidyl ethanolamine plasmalogen (Serdary Research Laboratories, Inc., London, Ontario, Canada); 1,10-decanediol and 1,12-dodecanediol (Koch-Light Laboratories, Colnbrook, Bucks, England).

Adrenal Specimens

Glands were removed at autopsy, usually within 48 hr of death, from a total of 61 Chinese adults and fetuses without adrenal disease. The adrenals were completely freed from the surrounding fat, weighed, and stored in chloroform (-20 C). Lipid extracts were prepared with chloroform-methanol (2:1) and washed with 0.0015 M $CaCl_2$ (6).

Colorimetric Methods

The estimation of *O*-alkyl and *O*-alk-1-enyl glycerol moieties in neutral lipid and phospholipid fractions (separated on short columns of silicic acid) was carried out on the glyceryl ethers liberated by means of saponification and isolated by means of thin layer chromatography (TLC); the phospholipid fractions were subjected to an additional step of phospholipase C digestion prior to saponification (7). The estimations of *O*-alkyl and *O*-alk-1-enyl glycerol moieties in individual lipid classes were carried out by the same procedures on ether-linked diacylglycerols, and ethanolamine and choline phosphoglycerides which were first isolated with the aid of TLC. Cholesterol was determined by the ferric chloride method (8).

O-Alkyl and *O*-Alk-1-enyl Glycerols

Total lipids (27.3 g from 350 g of adult adrenals) were separated into neutral lipids and phospholipids fractions by means of solvent partitioning (9). Alkyl and alk-1-enyl diacyl-

glycerols were partially purified from the neutral lipids fraction by column chromatography. A column (126 g silicic acid) prepared in diethyl ether-petroleum ether (60-80 C) in the proportions 1:99 was loaded with 2 g lipids and washed with a further 600 ml of the same mixture; the glyceryl ether diesters together with some triacylglycerols were eluted with 750 ml of diethyl ether-petroleum ether (1:24). Three such eluates were pooled and evaporated to dryness. The residue was saponified with 50 ml of 0.5 M KOH in methanol (50 C, 70 min). Seven volumes of water, methanol, and chloroform in the proportions 12:9:14 were shaken with the hydrolyzate. The lipids recovered from the chloroform layer were extracted with diethyl ether-petroleum ether (9:91) and the extract was applied to a column (18 g silicic acid) previously equilibrated with the same solvent mixture. The column was washed with 120 ml of the 9:91 mixture, followed by 240 ml diethyl ether-petroleum ether (1:3) and 300 ml diethyl ether. The diethyl ether eluate was evaporated to dryness, and the alkyl and alk-1-enyl glycerols contained therein were separated from each other and from other lipid components by TLC (diethyl ether-acetic acid, 99:1).

Ethanolamine and choline phosphoglycerides were isolated from the phospholipids fraction by means of preparative TLC (chloroform-acetone-acetic acid-water, 75:45:12:3). Chloroform-acetone-methanol-acetic acid-water (8:6:2:2:1) was used to test the purity of ethanolamine phosphoglycerides and chloroform-methanol-ammonia (65:25:5) for that of choline phosphoglycerides. Alkyl and alk-1-enyl glycerols were isolated from these fractions by means of a procedure utilizing phospholipase C, saponification, and TLC (diethyl ether-acetic acid, 99:1), and *O*-alkyl glycerols were isolated from total lipids from fetal adrenals by the same procedure (7).

Commercial sources provided the standard alkyl and alk-1-enyl glycerols for TLC and gas chromatography (GLC). Eicosanyl glycerol was prepared from its diacyl derivative by means of saponification and TLC. Standard *O*-alk-1-enyl glycerols were prepared from phosphatidyl ethanolamine plasmalogen (which was a mixture of *O*-alkyl acyl, *O*-alk-1-enyl acyl, and diacyl glycerophosphatides) by the previously specified phospholipase C-saponification-TLC procedure (7).

O-Alkyl glycerols and alkanediols were converted to their trimethylsilyl (TMS) ethers by reacting the dried lipid samples with Sil-Prep (22 C, 15 min). Diacetates of glyceryl ethers and of alkanediols were prepared by the method of Albro and Dittmer (10).

Chromatographic Methods

TLC was carried out on glass plates precoated with Silica Gel G with fluorescence indicator (Macherey Nagel, Düren, Germany). Plates used for the purification of glyceryl ethers were prewashed with chloroform-methanol (2:1). Silver ion-TLC plates were prepared before use by spraying prewashed plates with 10% silver nitrate in methanol-water (3:1) and drying them at 100 C (45 min). Lipids were located either by their fluorescence quenching property or by visualization with the aid of 2,7-dichlorofluorescein. The mercuric ion-dependent fuchsin reaction given by *O*-alk-1-enyl groups and the ninhydrin reaction were also employed where appropriate. Solvent systems used in TLC for the separation of ether-linked diacylglycerols were benzene-hexane-diethyl ether-acetic acid (45:50:5:1) or pure benzene. *O*-Alkyl glycerols were separated according to their degree of unsaturation by means of silver ion-TLC (11).

A Varian Aerograph model 2800 gas chromatograph with flame ionization detector was employed with 5 ft x 1/8 in. columns of 1.5% OV-101 on Chromosorb G and 10% OV-1 on Chromosorb W (220 C). Nitrogen flow rates (per min) were 30 ml for TMS-alkyl glycerols and diacetyl alk-1-enyl glycerols on OV-101; 12 ml for TMS-alkyl glycerols on OV-1; 45 ml for diacetyl alkyl glycerols on OV-101. The quantitative data presented on the composition of the alkyl and alk-1-enyl moieties were based on chromatograms obtained with OV-101 as the liquid phase. Theoretical plate numbers for the TMS ethers of octadecyl and octadecenyl glycerol were, respectively, 2300 and 2100; for the diacetyl derivatives of the corresponding alk-1-enyl glycerols, 1800 and 1700. Near baseline separations were obtained. The comparisons of the retention times of alkanediol and *O*-alkyl glycerol derivatives were obtained on a column of OV-101 (180 C; nitrogen, 25 ml/min).

RESULTS

Table I shows the lipid composition of representative adult and fetal adrenal specimens used in this study. The levels of cholesterol and other neutral lipid components varied markedly among the adult specimens, which were all taken from men (age 40-55 yr). This might have been expected since normal adrenals are rarely found post-mortem, and specimens taken routinely usually exhibit one of several types of lipid depletion (12). The lipid composition of fetal adrenals (28-36 wk) was less variable. On the average, fetal adrenals had significantly

TABLE I

Lipid Composition of Adult and Fetal Adrenal Specimens[a]

	Adult	Fetal	Difference[b]
Total lipids	101 ± 40 (27-142)	38 ± 15 (20-59)	P<0.05
Neutral lipids	87 ± 42 (6-130)	24 ± 13 (11-44)	P<0.05
Cholesterol	15 ± 21 (1-66)	7 ± 2 (4-9)	P<0.01
Phospholipids	14 ± 4 (9-21)	14 ± 4 (9-18)	NS
Neutral alkyl glycerols	58 ± 32 (10-102)	71 ± 61 (17-175)	NS
Neutral alk-1-enyl glycerols	42 ± 31 (4-93)	68 ± 53 (13-154)	NS
Alkyl phosphoglycerides	60 ± 48 (11-156)	61 ± 26 (39-107)	NS
Alk-1-enyl phosphoglycerides	205 ± 108 (89-380)	98 ± 46 (42-156)	P<0.05

[a]The values, expressed as mean ± SD (range), were based on analyses of 8 specimens from adults and 5 fetal specimens. The levels of total lipids, neutral lipids, cholesterol, and phospholipids are given in mg/g wet weight tissue; those of ether-linked glycerolipids in μg/g, computed as octadecyl or octadecenyl glycerol.

[b]Statistical significance of difference between the means obtained by means of the variance ratio test or Student's *t* test. P, probability level; NS, not statistically significant.

TABLE II

Composition of *O*-Alkyl and *O*-Alk-1-enyl Moieties in Ether-linked Glycerolipids of the Human Adrenal[a]

Side chain	Adult adrenals						Fetal adrenals
	Diacylglycerols		Ethanolamine phosphoglycerides		Choline phosphoglycerides		Total lipids
	Alkyl	Alk-1-enyl	Alkyl	Alk-1-enyl	Alkyl	Alk-1-enyl	Alkyl
16:1[b]	--	--	t[c]	t	t	t	t
16:0	6	9	52	52	42	61	16
17:1	t	t	t	t	t	t	--
17:0	t	t	t	t	t	t	1
18:1	21	17	15	20	47	16	50
18:0	17	26	33	28	11	15	15
19:1	4	--	--	t	t	8	--
19:0	t	--	t	--	t	--	3
20:1	4	5	--	--	t	t	2
20:0	4	7	t	--	t	--	2
21:1	t	--	--	--	--	t	--
21:0	t	--	--	--	--	--	t
22:1	16	14	--	--	t	--	4
22:0	7	7	t	t	t	--	4
24:1	21	15	--	--	t	--	3
24:0	t	--	--	--	--	--	--

[a]The 6 lipid fractions from adult adrenals were prepared from a single pool of tissues from 48 individuals. The total lipids of 7 fetal adrenal specimens were pooled in order to obtain the preparation of *O*-alkyl glycerols.

[b]The numbers before and after the colon refer to the number of carbon atoms in the side chain and the number of double bonds present in the chain at positions other than carbon 1, respectively.

[c]Trace component (t), amounting to less than 0.01 of the sample; identified solely on the basis of retention time. The figures denote area percentage, computed from gas chromatograms of the TMS ethers of *O*-alkyl glycerols and of diacetyl *O*-alk-1-enyl glycerols.

lower concentrations of alk-1-enyl phosphoglycerides, neutral lipids, and cholesterol. About half of the neutral alkyl glycerols in adult glands could be accounted for as alkyl diacylglycerols. Alk-1-enyl diacylglycerols were not detected in some specimens; in others, they amounted to almost half of the neutral alk-1-enyl glycerols present. The molar proportions of alkyl and alk-1-enyl glycerophosphatides in ethanolamine phospholipids were, respectively, 1.2 and 5.6%. Alkyl and alk-1-enyl phosphoglycerides comprised 0.9 and 1.8% of choline phospholipids.

The compositions of *O*-alkyl and *O*-alk-1-enyl moieties in several types of glycerolipids found in the adult adrenal are shown in Table II. These analyses were carried out on pooled specimens of adrenals taken from 48 individuals (age 59 ± 16 yr), the majority of whom were male. The *O*-alkyl and *O*-alk-1-enyl glycerols prepared from ether-linked diacylglycerols both contained significant proportions of compo-

TABLE III

Characterization of Glyceryl Ether Moieties in Ether-linked Diacylglycerols[a]

Assigned side chain composition	*O*-Alkyl glycerols (Trimethylsilyl ethers)	*O*-Alk-1-enyl glycerols (diacetates)
	Equivalent chain length (retention time, min)[b]	
16:0	16.00 (15.0)[c]	16.02 (16.5)
18:1	17.69 (25.4)[d]	17.73 (28.3)
18:0	18.02 (27.7)[c]	18.00 (30.8)
19:1	18.67 (34.2)[d]	18.74 (38.7)
20:1	19.68 (46.5)[d]	19.72 (52.3)
20:0	20.00 (51.0)[c]	20.00 (57.2)
22:1	21.71 (86.0)[d]	21.73 (96.4)
22:0	22.04 (94.5)[c]	22.04 (106)
24:1	23.72 (157.5)[d]	23.74 (178)

[a]Prepared from pooled adrenal tissues from 48 adults.

[b]Gas liquid chromatography on OV-101 as described under Methods.

[c,d]Found in the saturates (c) or the monoenes (d) fraction obtained by silver ion-thin layer chromatography.

nents with side chains having 20, 22, or 24 carbon atoms. These compounds included glyceryl ethers with a double bond at positions other than carbon 1, and they are represented in the table as 20:1, 22:1, and 24:1. The 22:1 and 24:1 groups, found in the *O*-alkyl glycerols fractions in the proportions of 16% and 21%, respectively, were also present in comparable amounts in the *O*-alk-1-enyl fraction. Glyceryl ethers with C_{20}, C_{22}, and C_{24} groups accounted for 52% of the total complement of alkyl glycerols and for 48% of the alk-1-enyl glycerols in ether-linked diacylglycerols. The commonly occurring 16:0, 18:1, and 18:0 groups were also found in these neutral glyceryl ether diesters. The ether-linked side chains in the phospholipid fractions consisted mainly of 16:0, 18:1, and 18:0 groups. Both the *O*-alkyl and *O*-alk-1-enyl fractions derived from ethanolamine phospholipids contained 52% of components with 16:0 groups and about 30% with 18:0 groups. The alkyl and alk-1-enyl glycerols derived from choline phospholipids contained different proportions of 16:0 and 18:1 groups, and the alk-1-enyl fraction also included a 19:1 component found only in trace quantities in the corresponding alkyl fraction.

O-Alkyl glycerols with long hydrocarbon side chains were also found in the total lipids of the fetal adrenals. Compounds with C_{19}, C_{20}, C_{22}, and C_{24} groups comprised 18% of the total complement of alkyl glycerols in the fetal gland (Table II).

The characterization of the individual glyceryl ether components in the adrenal lipids was obtained by a combination of GLC and TLC. The key observations are summarized in Table III. The equivalent chain length (ECL) of the individual alkyl glycerols was obtained from a plot of the log of the retention times of standard alkyl glycerols (14:0, 16:0, 18:0, and 20:0) versus the length of the side chains. The ECL of standard octadec-9-enyl glycerol (TMS ether) chromatographed on OV-101 was 17.67, indicating a fractional chain length (13) of about -0.3 for the monoene. Alkyl glycerols prepared from adrenal alkyl diacylglycerols and chromatographed in the same manner exhibited several components which had ECL corresponding to either saturates or monoenes. The assigned side chain compositions were confirmed by GLC of the saturates and the monoenes separated from a sample of the unknown alkyl glycerols by means of silver ion-TLC. The same complement of alkyl glycerols was also found by GLC on OV-1 of the TMS ethers and by analysis of the diacetyl derivatives on OV-101. The possibility that long-chain alkanediols might have been mistaken for some of the higher homologs of alkyl glycerols was ruled out by determinations of the ECL values of alkanediol derivatives, using the same procedure as that applied to the unknowns. The values, 8.16 (TMS ether) and 8.26 (diacetate) for 1,10-decanediol, 10.12 (TMS ether) and 10.17 (diacetate) for 1,12-dodecanediol, showed that the slopes of the log retention time-carbon number curves were different from those of the corresponding derivatives of *O*-alkyl glycerols; secondly, the apparent fractional chain length values for alkanediols were substantially different from those found for the various adrenal lipid components (Table III).

The unknown alk-1-enyl glycerols were identified by reference to the retention times of the diacetyl derivatives of alk-1-enyl glycerols prepared from beef brain ethanolamine plasmalogen, which have been identified and quan-

titated (10,14). The reference preparation was first standardized on OV-101 against the diacetyl derivatives of hexadecyl and octadecyl glycerol. The three major peaks in the chromatogram exhibited ECL values of 15.48 (area percentage, 32), 17.30 (41%) and 17.53 (27%), which were consistent with their identification as hexadec-1-enyl glycerol (represented in Table III as 16:0), octadec-1,9-dienyl glycerol (18:1), and octadec-1-enyl glycerol (18:0), respectively. A semilog plot of the retention times of these alk-1-enyl glycerol reference compounds versus the number of carbon atoms in the side chain was used to compute the ECL values shown in Table III.

DISCUSSION

The adult adrenal is composed of the medulla and the cortex, which differ in their embryological origin, structure, and function. Because of their close intermingling, it would have been difficult to obtain in quantity cleanly dissected cortical or medullary tissue. The higher levels of alk-1-enyl phosphoglycerides in adult glands, as compared to that in fetal adrenals, might be explained by their greater proportion of medullary tissue. The fetal adrenal is largely occupied by the fetal zone, which is probably the source of most of the lipids extracted from the fetal glands.

Alkyl and alk-1-enyl glycerol groups with 20 and 22 carbons have been found in the proportions of about 2% each in the ether-linked diacylglycerols of human perinephric fat (15). This is the first report of the co-occurrence of alkyl glycerol moieties with C_{24} groups in lipids from normal mammalian tissues. Such moieties were found in the alkyl diacylglycerols present in harderian gland tumors of mice (16). Saturated and unsaturated aldehydes with C_{24} groups have been isolated from human placental plasmalogens (17). Because transfer of certain lipids (such as dehydroepiandrosterone) between the placenta and the fetal adrenal is known to occur (18,19), there is the possibility that some of the ether lipids found in these two organs may not have been synthesized in situ. The presence of diacylglycerols with C_{22} and C_{24} alkyl and alk-1-enyl groups in the adult adrenal would indicate that the fetal adrenal at least had the latent ability to synthesize these unusual lipid components.

A pathway in mammalian cells for the synthesis of glycerolipids which contain ether bonds has been described (20). The ether bond is formed between a fatty alcohol and monoacyl dihydroxyacetone phosphate. The product, alkyl dihydroxyacetone phosphate, subsequently undergoes reduction, esterification at position 2, and dephosphorylation to form 1-alkyl-2-acyl-*sn*-glycerol. This compound can react with cytidine diphosphate- (CDP-) ethanolamine, CDP-choline, or acyl CoA. It is known that the resulting ethanolamine phosphoglyceride can undergo desaturation to yield the corresponding alk-1-enyl derivative (21). According to this scheme, the composition of the *O*-alkyl group in ether-linked diacylglycerols or glycerophosphatides would be determined at the step of synthesis of alkyl dihydroxyacetone phosphate. The observed differences in the distribution of the aliphatic moieties at position 1 in alkyl diacylglycerols, alkyl acyl glycerophosphoryl ethanolamines and alkyl acyl glycerophosphoryl cholines would suggest that they originated from different metabolic pools of 1-alkyl-2-acyl-*sn*-glycerol. Considering the complex structure of the gland, this result is not surprising. It is, of course, possible that the enzymes which add the radical at position 3 of 1-alkyl-2-acyl-*sn*-glycerol show preferences for certain *O*-alkyl groups on the substrate; so far this aspect of lipid synthesis has not received attention. The idea that glyceryl ether diesters and ether-linked phosphoglycerides do not share the same metabolic fate gains support from the fact that tissue levels of the former may vary independently in certain pathological conditions, namely, in malignancy (22) and in Wolman's disease (2).

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SHORT COMMUNICATION

Diester Waxes from Skin Lipids of the Feet of Biotin Depleted and Biotin Supplemented Turkey Poults

ABSTRACT

The neutral lipids of the skin from the feet of turkey poults fed a biotin supplemented or a biotin deficient diet consist mainly of triacylglycerols, and of mono- and diester waxes. Diester waxes from both groups were characterized as fatty acid esters of *erythro*-2,3-alkanediols. A comparison between fatty acid composition of the two groups, however, revealed the following significant differences. Biotin deficient birds showed a fairly high concentration of very long chain fatty acids (C_{36}-C_{40}) which were completely absent in biotin supplemented birds. Further, almost one-third of the fatty acids of diester waxes in biotin deficient birds were unsaturated while those from biotin supplemented birds were predominantly (96%) saturated.

INTRODUCTION

erythro-2,3-Alkanediol diesters have been reported to occur in the uropygial gland of chickan (1) and turkey (2). In a study primarily designed to define the effect of biotin deficiency in turkey poults, we have isolated and characterized these diesters from their cutaneous lipid. Although diesters of 1,2-alkanediols and 2-hydroxy fatty acids have been reported to occur in skin surface lipids (3), to our knowledge, the occurrence of 2,3-alkanediol diesters in an animal skin has not been reported before. The present paper gives the characterization and composition of these diesters derived from the skin of birds fed either a biotin supplemented [$Bio^{(+)}$] or a biotin deficient [$Bio^{(-)}$]diet.

MATERIALS AND METHODS

Broad-breasted white poults used for this study were fed the experimental diets from hatching at Hoffman-La Roche Inc., Nutley, NJ. The basic diet contained (per kg): sucrose, 412.5 g; vitamin-free casein, 170 g; isolated soy protein, 150 g; fish meal, 50 g; gelatin, 10 g; corn oil, 93 g; dicalcium phosphate, 30 g; non-nutritive fibre, 50 g; limestone, 5 g; methionine, 2 g; salt, 5 g; choline chloride, 2.5 g; vitamin and mineral supplements to provide: Vit. A, 15000 IU; Cholecalciferol, 3000 ICU; Vit. E, 25 IU; thiamin, 10 mg; riboflavin, 10 mg; pyridoxine, 8 mg; calcium panthothenate, 30 mg; niacin, 100 mg; folic acid, 0.4 mg; menaquinone sodium bisulfite, 4 mg; ethoxyquin 130 mg, cyanocabalamine 20 μg, magnesium 500 mg, manganese 60 mg, zinc, 58 mg; iron, 50 mg; potassium 3.6 g; copper, 5 mg; iodine, 1.25 mg; cobalt, 3.75 mg. Biotin deficient diet contained 28 μg d-biotin/kg feed; supplemented diet included 300 μg of additional d-biotin/kg feed. Birds were killed after 24 days. The feet and legs were removed, frozen in liquid nitrogen, and shipped on dry ice to Temple University. The skin from the legs and the dorsa of the feet were excised and freed from any traces of subcutaneous fat by scraping with prewashed glass wool. Extraction and purification were performed as published previously (4). $Bio^{(+)}$ and $Bio^{(-)}$ birds yielded ca. 40 mg and 32 mg, respectively, of lipid/g of skin. Reference *erythro*-2,3-alkanediols were isolated from uropygial glands of turkeys as described by Hansen et al (2). Acetonide and aldehyde standards were prepared from uropygiols by acetonation (1) and periodate oxidation (2), respectively. Infrared and nuclear magnetic resonance (NMR) spectra were recorded as described in (5). Gas liquid chromatography (GLC) was performed on F and M model 402 equipped with hydrogen flame ionization detector. Characterization of individual peaks was made by running the sample and reference compounds on OV-1 (4% on Gas-Chrom Q 100-200 mesh) and EGA (6% on Anakrom 90-100 mesh); by separating saturates and unsaturates by $AgNO_3$-silica gel thin layer chromatography (TLC) followed by GLC; and by analysis of the sample before and after hydrogenation. Quantitation was achieved by electronic integration.

RESULTS AND DISCUSSION

The lipid fraction (12% of total skin lipid) having a polarity in TLC (hexane-ether, 95:5)

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TABLE I

Relative Percentage[a] of Fatty Acid[b] and *erythro*-2,3-Alkanediols[c] of Diester Waxes Derived from Foot and Shank Skin Lipids of Turkey Poults Fed Biotin Deficient [$Bio^{(-)}$] or Biotin Supplemented [$Bio^{(+)}$] Diets

Carbon no.	Fatty acids		*erythro*-2,3-Alkanediols	
	$Bio^{(-)}$	$Bio^{(+)}$	$Bio^{(-)}$	$Bio^{(+)}$
12:0		4.9 ± .6		
14:0	9.1 ± .6	14.9 ± 1		
16:0	25.3 ± .6	31.0 ± 2	0.6	
16:1	2.5	tr[d]		
17:0	1.5 ± .3	1.8 ±..4		
18:0	19.0 ± 2	27.5 ± 1	0.7	1.5 ± .5
18:0[e]			0.8	
18:1	8.7 ± .6	1.5 ± .2		
18:2	7.9 ± .6	2.3 ± .2		
19:0	2.4 ± .4	3.7 ± .2	1.7 ± .2	0.5 ± .1
19:0[e]			0.31	
20:0	9.7 ± .7	11.7 ± .4	6.9 ± .3	10.6 ± .3
20:0[e]			0.6	
20:1	2.0 ± .4	tr[d]		
21:0			4.2 ± .2	4.2
21:0[e]			1.3	
22:0	0.9 ± .1		39.6 ± .8	62.8 ± 2
22:0[e]			0.6	
22:1	0.6			
23:0			5.9 ± 1	5.5 ± .1
24:0			14.3 ± 1	10.7 ± .3
25:0			0.5	0.5
25:0[e]			2.3 ± .1	0.4
26:0			2.3 ± .1	1.6 ± .1
27:0			17.8 ± 1	1.1
36:1	4.1 ± 1			
37:1	1.4 ± .3			
38:1	3.3 ± .3			
39:1	tr[d]			
40:1	1.6 ± .3			

[a]Average ± SD for three animals.

[b]As methyl esters.

[c]As acetonides.

[d]Tr = detectable amounts that represent less than 0.25%

[e]Branched.

between that of wax esters and triacylglycerols was characterized as a mixture of *erythro*-2,3-alkanediol diesters by spectroscopic and chemical methods. The infrared spectrum of the material exhibited the presence of ester carbonyl (1743 cm^{-1}), but no characteristic absorption for a glyceryl ether function was observed in the region of 1110-1132 cm^{-1} (6). The NMR spectrum showed characteristic features of 2,3-alkanediol diesters (7). A doublet at δ 1.12 ($C\underline{H}_3CHOCOR$, $\underline{J}$=7 Hz) and a multiplet at 4.9 ppm assigned to 2- and 3-position methylene protons of the glycol moiety were found to be coupled to each other. The esters were further characterized by hydrolyzing with methanolic KOH and separating the acidic and neutral products on an alkaline silicic acid column (8). The neutral component appeared as a uniform spot in TLC, having an Rf value identical to that of *erythro*-2,3-alkanediols on boric acid impregnated silica gel (2). GLC of the acetonides of these diols showed identical retention times to those of reference acetonides, and further indicated the absence of *threo* isomers. Metaperiodic acid oxidation of the diols yielded aldehydes of expected carbon numbers, which showed identical Rf value (0.66, hexane-ether, 80:20) and retention times to those of reference aldehydes. The fatty acid and diol compositions of these diesters is shown in Table I. Comparison of these results with the reported composition of diesters of turkey preen gland (2) revealed significant differences: $C_{18:0}$ constituted the most predominant acid in diesters of uropygial gland, while $C_{16:0}$ was the most abundant fatty acid in skin diesters; unsaturated fatty acids did not occur in uropygial gland (2), while a significant amount of these was present, particularly in skin lipid of $Bio^{(-)}$ birds; in the diols, $C_{21:0}$

was reported to be the second most predominant in uropygial gland (2) but was not present in any significant amount in skin of $Bio^{(+)}$ or $Bio^{(-)}$ birds; and the cutaneous diesters showed a fairly high concentration of diols having chain lengths longer than C_{23}, which were not present in diesters from the uropygial gland.

A comparison between $Bio^{(+)}$ and $Bio^{(-)}$ birds is also of great interest. Surprisingly, biotin-deficient birds showed a fairly high concentration of very long chain fatty acids which were completely absent in birds fed a biotin supplemented diet. Another striking difference is that fatty acids of $Bio^{(+)}$ birds were largely saturated, while almost one third of fatty acids from biotin deficient birds were unsaturated. Whether this difference in fatty acid and diol composition can be ascribed specifically to biotin deficiency is under further investigation.

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Metabolism and Tissue Distribution of Label from [9,10-methylene-^{14}C] Sterculic Acid in the Rat[1,2]

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ABSTRACT

The metabolism of ^{14}C-sterculic acid, labeled in the methylene carbon of the cyclopropene ring, was investigated in Wistar rats. Comparison of the distribution of radioactivity in tissue and excreta as a function of time showed that the free sterculic acid was metabolized faster than the methyl ester and that the sterculic acid administered by intragastric intubation was absorbed and metabolized at a faster rate than that administered by intraperitoneal injection. The concentration of label in blood serum reached a maximum 2 hr after intubation and then rapidly declined. Incorporation of radioactivity into most organs peaked at 4 hr with liver peaking at a maximum of 11% of the administered dose and other organs at less than 1%. Label in depot fat steadily increased to 8% at 26 hr. Less than 1% of the administered dose was expired in CO_2 in the same time period. Excretion of label reached a maximum of 48% in urine and 11% in feces by 16 hr. The majority of the label in liver was in the fatty acid portion of the lipid fraction. The relative amount of label in microsomal and mitochondrial subcellular fractions of liver changed with time suggesting that these organelles may be involved in the metabolism of sterculic acid. Rats fed control diets appeared to metabolize sterculic acid in the same manner, but at a slower rate than rats acclimated to dietary cyclopropene fatty acids. Low recovery of label in expired air showed that the methylene carbon of the cyclopropene ring was not oxidized to CO_2. These data suggest that rats readily absorb sterculic acid and excrete labeled compounds primarily in the urine.

INTRODUCTION

Sterculic acid (SA) is a unique naturally occurring cyclopropenoid fatty acid (CPFA) containing a highly strained and reactive unsaturated three membered ring in the center of an eighteen carbon chain. Cottonseed oil, used extensively in the U.S. as a food oil, and kapok oil, an important oil in the oriental countries, contain various amounts of CPFA (1). Oils possessing this small ring are held responsible for certain physiological disorders in animals (2-4). Cyclopropene fatty acids were first reported to be co-carcinogenic (5) and then demonstrated to be carcinogenic in trout (6).

Metabolic studies of SA have been limited. Altenburger and co-workers (7) administered [9,10-methylene-^{14}C]sterculic acid intravenously to fasting hens and found limited amounts of $^{14}CO_2$ formed over a 24 hr period with more than 50% of the label excreted in the fecal material (feces and urine). They concluded that very little of the labeled sterculic acid had been metabolized. Other studies with dihydrosterculate (8) noted that short chain acids with the cyclopropane ring intact accumulated in adipose tissue.

The purpose of this investigation was to study the metabolism of [9,10-methylene-^{14}C]sterculic acid in the rat. The study was divided into three parts: experiment I was designed to indicate which derivative of the carbon-14 label, SA or methyl sterculate (MS), and which mode of administration of the label, intraperitoneal (IP) or intragastric (IG), were best suited for rat metabolic studies. Experiment II was designed to study the distribution of ^{14}C from [9,10-methylene-^{14}C]sterculic acid in the various rat organs and tissues over various time periods, and experiment III was designed to compare the incorporation of ^{14}C from [9,10-methylene-^{14}C]sterculic acid into liver fractions and urine from rats fed corn oil with rats fed *Sterculia foetida* oil.

MATERIALS AND METHODS

Source of Label

Methyl sterculate, labeled in the 9,10-methylene bridge of the cyclopropene ring, was synthesized in our laboratory (9) and shown to be 96.8 ± 2.5% and 99.0 ± 2.0% cyclopropene by the Halphen Test (10) and nuclear magnetic resonance (NMR) (11), respectively. The methyl sterculate had a specific activity of 110 μci/mmole and was radiochemically pure when analyzed by thin layer chromatography (TLC)

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TABLE I

Distribution of Radioactivity in Rats Injected Intraperitoneally (IP) and Intragastrically (IG) with Carbon-14 Labeled Methyl Sterculate and Sterculic Acid

	Percent of administered carbon-14 activity[a]					
	Methyl sterculate			Sterculic acid		
	6 hr, IP	12 hr, IP	6 hr, IG	6 hr, IP	12 hr, IP	6 hr, IG
Stomach	0.1	0.2	7.7	0.5	0.8	6.8
Intestine	0.7	0.1	47.5	7.1	18.9	19.5
Liver	0.4	1.0	8.0	2.6	8.2	9.3
Spleen	0.2	0.5	0.1	0.0	0.1	0.1
Heart	0.1	0.0	0.3	0.0	0.1	0.2
Lung	0.5	0.0	0.1	0.1	0.1	0.1
Testes	0.1	0.3	0.0	0.1	0.6	0.1
Kidney	0.2	0.0	0.8	0.3	0.6	0.6
Urine	0.2	0.4	4.2	6.7	43.0	29.3
Feces	0.1	2.9	0.4	0.0	4.9	0.0
CO_2	0.1	0.0	0.1	0.3	0.6	0.4
Total Recovered[b]	—[c]	40.6	82.2	26.0	91.4	83.3

[a]One rat was used for each time period.

[b]Activity recovered in organs listed in table plus carcass. The low recovery in rats dosed IP was caused by unabsorbed radioactivity in the peritoneal cavity that was lost when the organs were removed.

[c]Radioactivity in carcass was not determined.

in a hexane-ether-benzene (70:30:1) system. The label migrated as a single spot coincident with the methyl sterculate at a R_f of 0.60. The methyl ester was converted to the free acid by saponification with 0.5N 95% ethanolic KOH at 45-50 C for 1.5 hr and shown to be 93.2 ± 1.1% and 96.7 ± 2.0% cyclopropene by the Halphen Test and NMR, respectively.

Experiment I — Mode of Administration of Label

Male Wistar strain rats weighing 251 ± 8 g and fed rat chow were starved 12 hr before intraperitoneal or intragastric administration of 4.17 ± 0.45 μci of labeled MS or SA in corn oil. Immediately after injection, the animals were placed in metabolic chambers. Air was pulled through two 80 ml KOH traps to collect $^{14}CO_2$. Feces were collected on wire mesh screens and urine flowed into glass tubes packed in ice.

At 6 or 12 hr after injection, the animals were decapitated. Blood was collected and immediately centrifuged. Organs and tissue were removed, weighed, wrapped in aluminum foil, and stored at -10 C, along with the feces, urine, carcass, and serum. Serum protein was determined by the Lowry method (12).

Organs, tissue, and feces were homogenized in 3 or 4 volumes (w/v) distilled water with a Tissumizer SDT 100N (Tekmar Company, Cincinnati, OH). Aliquots of the homogenates were digested in NCS tissue solubilizer (Amersham/Searle, Arlington Heights, IL) and counted in Aquasol (Aquasol, Nuclear-Chicago, Des Plaines, IL) and corrected for quenching. The frozen carcass was ground in a meat grinder and digested in 300 ml 6N KOH. Aliquots of the digested carcass, Urine, serum, and KOH traps were also counted in Aquasol.

Experiment II — Deposition and Excretion of Label

Two male Wistar strain rats, weighing 252 ± 8 g, were injected intragastrically with 5.66 ± 0.78 μci of SA in corn oil, and immediately placed into metabolic chambers for each of the following time periods: 1, 2, 4, 8, 16, and 16 hr. Blood, organs, carcass, excreta, and KOH traps were analyzed as described in experiment 1.

As quickly as possible, livers were minced in four volumes (w/v) of 0.25M ice-cold sucrose and homogenized in a Potter-Elvehjem homogenizer with eleven up and down strokes of a loosely fitting teflon pestle. A model L-2 Ultracentrifuge (Beckman, Inc., Palo Alto, CA) was used to prepare liver subcellular fractions of 10 ml aliquots of the homogenates. The cell debris, mitochondrial, microsomal, and supernatant fractions were prepared by spinning at 800 x g for 10 min, 12,000 x g for 10 min, and 105,000 x g for 60 min, respectively. Each sediment was washed once with 0.25M sucrose and the wash was added to the supernatant before the next fraction was sedimented. Aliquots of the liver homogenate and the subcellular fractions were counted in Aquasol.

TABLE II

Percent of Administered Radioactivity Recovered in Tissues and Excreta of Rats after Intubation with [9,10-methylene-^{14}C] Sterculic Acid

	Hours[a]					
	1[b]	2	4	8	16	26
Stomach	47.4	25.9 ± 5.2	14.2 ± 0.19	1.17 ± 0.19	0.20 ± 0.18	0.33 ± 0.16
Small intestine	50.7	33.2 ± 7.5	25.8 ± 8.0	6.34 ± 0.85	1.94 ± 1.69	2.34 ± 0.07
Liver	4.30	5.90 ± 3.37	10.9 ± 1.6	8.28 ± 0.77	6.13 ± 0.12	2.56 ± 0.20
Kidney	0.22	0.39 ± 0.22	0.78 ± 0.13	0.58 ± 0.12	0.46 ± 0.13	0.27 ± 0.00
Fat	3.85	4.33 ± 2.16	3.38 ± 0.63	4.29 ± 0.56	5.75 ± 0.84	7.78 ± 2.11
Ceacum	0.04	1.92 ± 1.65	5.82 ± 0.19	7.51 ± 0.14	4.08 ± 1.40	4.67 ± 0.68
Colon	0.14	1.76 ± 1.36	0.26 ± 0.04	3.81 ± 1.13	1.63 ± 0.02	5.07 ± 0.27
Feces	0.00	0.00	0.00	0.20 ± 0.07	11.1 ± 0.8	5.71 ± 2.16
Urine	0.42	2.19 ± 1.14	8.89 ± 4.52	23.9 ± 3.6	48.3 ± 3.6	47.9 ± 4.0
CO_2	0.00	0.00	0.00	0.00	0.36 ± 0.00	1.04 ± 0.05
Blood[c]	7.3	11.6 ± 2.8	5.8 ± 1.4	3.6 ± 1.4	0.8 ± 0.6	0.6 ± 0.1

[a]Data expressed as the average of two rats ± the range.

[b]Data from one rat.

[c]Radioactivity in blood expressed as dpm per mg serum protein divided by the dpm administered per mg body weight.

Experiment III – Distribution of Label in Liver and Urine from Corn Oil and *Sterculia foetida* Oil Fed Rats

Weanling Wistar strain male rats were fed ad libitum a Kirshnaro and Draper diet (13) modified to contain 20.0% protein and 32.2% cornstarch. The diet, consisting of 92.8% dry ingredient premix, 2.2% vitamin mix (U.S. Biochemicals, Cleveland, OH), and 5.0% corn oil, was mixed 1:1 (w/v) with 3.0% agar (DIFCO) to make a solid gel. After 2 wk on the control diet, half of the rats were transferred to a 4.0% corn oil (CO) plus 1.0% *Sterculia foetida* oil (SFO) diet. Rats were acclimated to this diet for at least 60 days.

Animals were paired, one CO fed and one SFO fed, administered SA by gavage, and placed in metabolic chambers. Carbon dioxide and urine were collected for 10 hr. The animals were decapitated and the livers fractionated according to the method of Shibko and coworkers (14) into three fractions: glycogen fraction (acid soluble compounds), lipid fraction, and protein fraction. The lipids, were saponified and fractionated into three more fractions (15): nonsaponified steroid fraction (hexane nonpolar soluble), fatty acid fraction (ethyl ether soluble), and glycerol fraction (water-alcohol soluble). Aqueous samples were counted in Aquasol and samples in nonpolar solvent were counted in toluene containing 6 g PPO (2,5-diphenyloxazole, Sigma Chemical Co.) plus 50 mg POPOP (1,4-bis-[2,5-phenyloxazole] benzene, Nuclear Chicago) per liter.

RESULTS AND DISCUSSION

Experiment I

Compounds administered by different routes may produce different pharmacological responses (16) and may be metabolized to different derivatives (17). Because of these variables, such as absorption and transportation, an initial study was done to determine the route of administration and form of SA best suited for the deposition and distribution studies in the rat.

Intraperitoneal injection of MS, shown in Table I, was a poor mode of administration. Very low levels of carbon-14 label were incorporated into organs and excreta at 6 or 12 hr. Intragastric administration of MS yielded a higher incorporation of label into organs and excreta with the urine containing 4.2% of the label after 6 hr. However, a slow rate of absorption was indicated since 55.2% of the administered dose was still in the stomach and small intestine after 6 hr.

Carbon-14 labeled SA produced higher levels of label in rat organs and excreta than MS for both intraperitoneal or intragastric administration. Six hours after the intraperitoneal and intragastric administration of SA, 6.7% and 29.3% of the label was recovered in the urine. The overall higher levels of label in urine and organs indicated that SA was absorbed, transported, and metabolized much faster than MS. Thus, SA was used in the following experiments.

Intragastric intubation was the preferred route of administration because rapid metabolism of SA occurred by this route and oral consumption is the common mode of exposure to CPFA.

Experiment II

The distribution of radioactivity was de-

TABLE III

Percent of Administered Radioactivity Recovered in Liver Subcellular Fractions from Rats after Intubation with[9,10-methylene-^{14}C]Sterculic Acid

Subcellular fractions	Hours[a]					
	1[b]	2	4	8	16	26
800 x g cell debri	0.74	1.18 ± 0.73	3.10 ± 0.58	2.69 ± 0.22	2.51 ± 0.002	1.01 ± 0.01
12,000 x g mitochondrial	0.28	0.59 ± 0.30	0.96 ± 0.11	1.35 ± 0.13	0.61 ± 0.04	0.37 ± 0.06
105,000 x g microsomal	0.26	0.71 ± 0.34	1.13 ± 0.11	0.67 ± 0.18	0.35 ± 0.05	0.17 ± 0.02
supernatant	0.91	2.46 ± 1.02	5.29 ± 0.51	3.67 ± 2.09	2.45 ± 0.03	1.06 ± 0.10

[a]Data expressed as the average of two rats ± range.

[b]Data from one rat.

termined at various times after the rats were dosed intragastrically with [9,10-methylene-^{14}C] sterculic acid. The radioactivity recovered in a particular tissue or organ was expressed as the percentage of the total administered dose. After 2 hr, 26% of the label was found in the stomach (Table II) and after 8 hr about 1% was left. Table II shows that by 8 hr about 94% of the administered label has been absorbed or passed from the small intestine and by 16 hr about 98% had been removed. The small intestine is the organ normally involved in the absorption of fatty acids (18).

Between 4 and 8 hr, the amount of label in the small intestine decreased and the amount of label in the colon increased to about 4% (Table II). At 26 hr, the colon contained about 6% of the label and the ceacum about 5%. Hence, about 5 to 6% of the administered label was not absorbed by the small intestines but was passed on to the colon and excreted as indicated by an increase in fecal radioactivity at 16 hr. The urine activity reached a maximum of 48% at 16 hr with no decrease at 26 hr (Table II). Only 1% of the administered carbon-14 label was trapped in CO_2 of expired air during the 26 hr period (Table II). Carbon-14 activity in the liver and kidney reached a maximum of 11% and 1%, respectively, at 4 hr. Less than 0.6% of the activity was found in the spleen, heart, lung, and testes at any time period. The rat can store cyclopropenes in the fatty tissue as evidenced by recovery of 8% of the label in the fat at 26 hr (Table II). Deposition of CPFA into body fat has been reported (2,3).

The concentration of label in the blood serum expressed as a function of time is shown in Table II. The peak concentration occurred at 2 hr with a rapid decrease at longer time periods. This would indicate a relatively fast transfer of label from the small intestine to and from the liver with a minimum of enterohepatic circulation.

Excretion of label in the urine leveled off at 26 hr (Table II) after 48% of the label had been excreted. Table II shows that 59% of the administered dose was excreted in the urine and feces after 16 hr. The high concentration of label in urine indicates that ^{14}C from SA was incorporated into water soluble compounds that were excreted primarily in the urine. Furthermore, the low concentration of label in expired CO_2 suggests that the labeled carbon-14 methylene group was not oxidized to CO_2.

The distribution of label in liver subcellular fractions is shown in Table III. High activity in the supernatant was probably due to the movement of SA or metabolites of SA between organelles in the cytosol. The microsomal and mitochondrial fractions both contained a significant amount of label after 4 hr. The time of peak concentration of label in the microsomal fraction, 4 hr, came before the time of peak concentration of label in the mitochondrial fraction, 8 hr. Variations in the relative concentration of radioactivity in the different fractions as a function of time suggest that various phases of SA metabolism may be catalyzed by different subcellular fractions.

Experiment III

Rats fed the CO and 1% SFO diets for 60 days were healthy and had similar body weight gain. The distribution of label in the CO fed rats for the 10 hr period was comparable to that in experiment II. Table IV shows the distribution of label in the various components and fractions expressed as a percent of the administered labeled dose. Most of the radioactivity was excreted in the urine over the 10 hr period. The SFO fed rats excreted approximately 2.5 times more label in the urine than CO fed rats suggesting that SFO fed rats metabolized the SA much faster than controls. The labeled CO_2 accounted for less than 1% of

TABLE IV

Radioactivity in Various Tissue Fractions of Corn Oil and *S. foetida* Oil fed rats after Intubation with [9,10-methylene-^{14}C] Sterculic Acid[a]

Diet	Corn oil[b]	*S. foetida* oil[b]
Liver	7.27 ±3.15	7.77 ± 4.77
Urine	20.17 ± 27.29	51.76 ± 7.47
CO_2	0.64 ± 0.58	0.55 ± 0.15
Liver Fractions		
Protein	0.57 ± 0.33	0.23 ± 0.16
Lipid	5.89 ± 2.98	7.16 ± 4.52
Acid soluble	0.81 ± 0.16	0.36 ± 0.09
Liver Lipid Fractions		
Steroid	c	c
Fatty acids	5.89 ± 2.98	7.16 ± 4.52
Glycerol	c	c

[a]Expressed as % of administered dose of [9,10-methylene-^{14}C] sterculic acid after 10 hr.

[b]Data expressed as average of two rats.

[c]Activity was less than 0.01% of dose.

the activity in both groups. Very little of the ^{14}C from labeled SA was found in compounds other than urinary compounds in both CO and SFO fed rats. In the liver, the majority of the label (5.89%) was in the lipid fraction in the fatty acids. Only a small amount of the label (0.24-0.57%) was bound to proteins, possibly to sulfhydryl groups as suggested by Kircher (19). The acid soluble fraction (0.36- 0.81%) probably containted ^{14}C compounds which had not been transported from the liver.

The label in rat urine could be extracted only under acidic conditions. Two extractions with 100 ml of ethyl ether of urine acidified to pH 1-2 with HCl yielded a maximum recovery of 88-90% labeled for both CO and SFO fed rats. Additional extraction of urine with ethyl ether did not improve the yield. Thus, about 10% of label was probably excreted as conjugated derivatives. However, attempts to release extractable metabolites in urine by treatment with glucouronidase were unsuccessful (20).

The data reported in this paper demonstrate that rats readily absorb ^{14}C-sterculic acid and excrete the label in the urine. The metabolism occurred primarily in the liver and did not involve oxidation of the methylene carbon of the cyclopropene ring to CO_2. Acclimation of rats to cyclopropene fatty acids did not change the pattern of excretion but appeared to increase the excretion of ^{14}C in the urine. The identification of the urinary ^{14}C-labeled compounds is the subject of a separate communication (21).

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The Preparation, Gas Liquid Chromatography, and Some Physical and Chemical Properties of Certain Higher Aliphatic 2,4-Diketones – A New Lipid Class

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ABSTRACT

Fourteen saturated aliphatic 2,4-diketones, ranging in chain length from C_7 to C_{25}, and one unsaturated compound, Δ^{12}-2,4-heneicosenedione, were prepared by Claisen acylations of the appropriate methyl ketones, and their gas chromatographic behavior was studied. Mixtures of these diketones were resolved on columns containing UCW98 or Carbowax 20M stationary phases. Their methoxime and 2,4-dinitrophenylsemicarbazone derivatives were resolved on OV-17 and OV-1 stationary phases, respectively. Since higher homologous aliphatic 2,4-diketones have been found in mammalian tissues, they constitute a new lipid class.

INTRODUCTION

A group of homologous aliphatic 2,4-diketones with chain lengths ranging from C_{13} to C_{25} has been found widely distributed in mammalian tissues. These compounds, which comprise a new lipid class, are apparently derived in vivo from the naturally occurring fatty acids through the condensation of a molecule of fatty acid with a molecule of acetone:

$$RCOOH + CH_3COCH_3 \rightarrow RCOCH_2COCH_3$$

where $R = CH_3(CH_2)_n$ and n = 8—20

It appears that unsaturated chains may also occur; however, the saturated compounds, with n even, are the more abundant. The inhibition of the action of histamine on smooth muscle by tissue extracts (1-4) has been attributed by us to the presence of these 2,4-diketones in such extracts. Details concerning the isolation of this 2,4-diketone fraction from tissues, its gas liquid chromatographic resolution, and the confirmatory identification of individual components will be presented in another publication. This communication deals with the synthesis of a number of 2,4-diketones which were required for reference purposes and for pharmacological investigations, their gas liquid chromatographic behavior, and with some physical and chemical properties of these compounds.

Syntheses of various homologous saturated 2,4-diketones have been reported in the literature; they were usually prepared by a Claisen condensation between the appropriate methyl ketone and ethyl acetate in the presence of sodium (5-8), sodamide (9), or sodium hydride (10), or, alternatively, by the condensation of a fatty acid ethyl ester with acetone in the presence of one of these condensing agents. Several 2,4-diketones have been synthesized as well by the reaction of acetoacetyl chloride with alkylmagnesium bromides (8).

For this present investigation, a few 2,4-diketones, up to 2,4-tetradecanedione, were conveniently prepared by a Claisen condensation between the appropriate commercially available methyl ketone and acetone in anhydrous diethyl ether, with sodium hydride as the condensing agent (10); for the preparation of higher homologues, the required methyl ketones were synthesized by the acylation of ethoxymagnesiomalonic ester with the appropriate fatty acid chloride, followed by acidolysis of the resulting acylmalonic ester to the desired methyl ketone (11,12).

The 2,4-diketones, which were thus synthesized, were amenable to gas liquid chromatography without prior derivatization. Of the several stationary phases, including OV-1, PPE-20, UCW98, and Carbowax 20M, which were investigated, UCW98 was found to be satisfactory. Most of our work was done with columns packed with 10% UCW98 on Diatoport S or Chromosorb W-AW (DMCS) which were operated isothermally.

2,4-Dinitrophenylhydrazones and -semicarbazones (13,14) of several 2,4-diketones were prepared. The former were viscous oils and were not investigated further. The 2,4-dinitrophenylsemicarbazones were chromatographed on a 3% OV-1 column operated at 200 C. Methoxime derivatives (15,16) of 2,4-diketones were resolved on a 3% OV-17 column at 230 C.

[1] Deceased.

MATERIALS AND METHODS

Sodium Hydride

Sodium hydride, 50% in oil, was purchased from British Drug Houses. Just prior to use, the oil was removed by washing the weighed sample of NaH with petroleum ether (30 C-60 C) in the reaction flask.

Methyl Ketones

2-Pentanone, 2-heptanone, 2-octanone, 2-undecanone were purchased from J.T. Baker Chemical Co. and Anachemia Chemicals Ltd., Montreal and were of practical or laboratory grade.

Fatty Acids

These were of laboratory grade. Pelargonic acid was synthesized from 1-bromooctane and sodium cyanide.

Florisil

Florisil (60-100 mesh), Floridin Company, Tallahassee, FL, was treated with 1:1 hydrochloric acid, washed repeatedly with distilled water, and then was dried at 120 C and activated at 300 C overnight.

Gas Liquid Chromatography (GLC)

A Hewlett-Packard Model 5750 gas chromatograph, with dual columns and dual flame ionization detectors, was employed. For the chromatography of 2,4-diketones, a 6 ft x 1/8 in. stainless steel column, packed with 10% UCW98 on Diatoport S or Chromosorb W-AW (DMCS), was used. It was operated isothermally at 200 C, 230 C, or 240 C. Injection port and FID temperatures were 240 C and 260 C, respectively, and the helium flow rate was 30 ml/min. Mixtures of 2,4-diketones were separated also with a 6 ft x 1/8 in. stainless steel column packed with 4% Carbowax 20M on Diatoport S, and operated at 210 C or at 220 C. For the methoximes, a 6 ft x 1/8 in. stainless steel column, containing 3% OV-17 on Chromosorb W, was employed. The 2,4-dinitrophenylsemicarbazones were separated on a 30 in. x 1/4 in. glass column packed with 3% OV-1 on Gas Chrom Q.

Ultraviolet Spectrophotometry

A Beckman DK-2 Ratio-Recording Spectrophotometer was employed.

Infrared Spectrophotometry

Infrared spectra were obtained with a Perkin-Elmer Infracord. Samples were mounted as liquid films between sodium chloride discs.

Melting Point Determinations

A Kofler micro-melting point apparatus, attached to the stage of a microscope, was employed. Eutectic melting points of mixtures of several crystalline 2,4-diketones with azobenzene have been recorded as well.

Syntheses of 2,4-Diketones

The following 2,4-diketones were synthesized by the condensation of the appropriate methyl ketone with ethyl acetate in the presence of sodium hydride (10): 2,4-heptanedione, 2,4-nonanedione, 2,4-decanedione, 2,4-tridecanedione, and 2,4-tetradecanedione. 2,4-Octadecanedione was prepared from 2-hexadecanone which was obtained by the oxidation of 2-hexadecanol. The diketones were purified via their copper chelates, and the lower members were distilled in vacuo.

The following 2,4-diketones were synthesized from methyl ketones which were prepared from the acid chlorides of the appropriate fatty acids (11,12): 2,4-dodecanedione, 2,4-pentadecanedione, 2,4-heptadecanedione, 2,4-nonadecanedione, 2,4-eicosanedione, 2,4-heneicosanedione, Δ^{12}-2,4-heneicosenedione, 2,4-tricosanedione, and 2,4-pentacosanedione.

Three typical syntheses are summarized below. Full details regarding the preparation of the compounds mentioned above will be provided to interested parties on request.

Synthesis of 2,4-Nonadecanedione

2-Heptadecanone was prepared from palmitoyl chloride and ethoxymagnesiomalonic ester (11,12). The 2-heptadecanone was distilled at ca. 0.1mm Hg in a Hickman still. The yield, based on the palmitoyl chloride, was 58%. 2-Heptadecanone (0.05 mole) in anhydrous diethyl ether was added to a stirred mixture of ethyl acetate (0.1 mole) and sodium hydride (0.05 mole) in anhydrous ether. After completion of the reaction, the crude 2,4-nonadecanedione was recovered following acidification of the reaction mixture. After purification of the product via the copper chelate (17), a 41% yield of 2,4-nonadecanedione, based on 2-heptadecanone, was obtained. The mass spectrum has a parent peak at m/e 296. The calculated molecular weight for $C_{19}H_{36}O_2$ is 296.4.

Synthesis of Δ^{12}-2,4-Heneicosenedione

Oleic acid was converted to oleoyl chloride with oxalyl chloride (18). Oleoyl chloride was allowed to react with ethoxymagnesiomalonic ester. Following acidolysis of the acylmalonic ester (11), the crude Δ^{10}-2,4-nonadecenone was purified on a Florisil column followed by

distillation under high vacuum. The product exhibited IR absorption maxima at 2860, 1690, 1440, 1340, and 1160 cm^{-1}; this spectrum was similar to that of a reference methyl ketone, 2-heptanone. The Δ^{10}-2-nonadecenone was condensed with ethyl acetate in the presence of sodium hydride to form Δ^{12}-2,4-heneicosenedione. The latter compound was purified by the formation of its copper chelate with cupric acetate in aqueous ethanol, followed by regeneration of the diketone with dilute acid. The yield was 29.5% from the methyl ketone. A pure sample of the diketone was obtained by preparative GLC on a 3% OV-1 column. The mass spectrum has a parent peak at m/e 322. The calculated molecular weight for $C_{21}H_{38}O_2$ is 322.3. The IR absorption spectrum exhibited maxima at 2900, 2820, 1680 (weak), 1600, 1350, 1230, 960, 770, and 750 cm^{-1}.

Synthesis of 2,4-Pentacosanedione

Starting with docosanoic acid, which was converted to the acid chloride by means of thionyl chloride, 2,4-pentacosanedione was prepared by the procedure outlined above for 2,4-nonadecanedione. The yield was 47% based on docosanoic acid. The product was purified by preparative GLC on 3% OV-1 column at 240 C. The mass spectrum had a parent peak at m/e 380. The calculated molecular weight for $C_{25}H_{48}O_2$ is 380.6.

Preparation of Methoxime and 2,4-dinitrophenylsemicarbazone Derivatives of 2,4-Diketones

The methoxime derivatives were prepared by heating various 2,4-diketones with an excess of ca. 2.5% methoxyamine hydrochloride in pyridine for 3 hr at 60 C (16). The reaction mixture was diluted with water and the mixture was extracted with petroleum ether. The organic phase was washed with 1N hydrochloric acid, then with saturated aqueous sodium bicarbonate, was dried and evaporated.

The 2,4-dinitrophenylsemicarbazones were prepared as described by Tumlinson et al. (14).

Thin Layer Chromatography (TLC) of 2,4-Diketones

On Bakerflex Silica Gel Film IBF with chloroform as the developing solvent, 2,4-diketones with chain lengths from C_5 to C_{23} had R_f values between 0.4 and 0.5. The compounds were located by their ultraviolet absorption or by their reaction with 2,4-dinitrophenylhydrazine. TLC thus had a potential application only in separation of the 2,4-diketones as a group. Great care had to be exercised to avoid the inadvertent formation of chelates with di- and trivalent cations which may be present as impurities in some silica gel preparations. Silica Gel G is useless, as it contains calcium sulfate as a binder.

Alkaline Hydrolysis of 2,4-Diketones

An 0.1 mg sample of the 2,4-diketone was placed in a 7 ml vial and 0.2 ml 5% aqueous potassium hydroxide was added. The vial was closed with a tightly fitting Vacutainer stopper, and it was heated at 80 C in a heating block overnight. A 1.0 ml sample of the headspace was withdrawn with a 2.5 ml gas-tight syringe and injected onto a Porapak Q column (6 ft x 1/8 in., stainless steel) operated at 130 C (19). Confirmation of the acetone peak was made by headspace analysis of an aqueous solution of acetone which had been treated in the same manner as the sample. The fatty acid was extracted from the aqueous solution in the vial after acidification of the contents and was identified as the methyl ester by GLC on a 10% UCW98 column.

Periodate-Permanganate Oxidation of 2,4-Diketones

Samples of 2,4-diketones were oxidized with the von Rudloff periodate-permanganate reagent (20) as described by Chang and Sweeley (21) with the modification that the tert-butanol was omitted. The carboxylic acids resulting from the reaction were identified as their methyl esters by GLC.

RESULTS AND DISCUSSION

Satisfactory yields of 2,4-diketones were obtained by the method outlined in the previous section. Attempts to condense higher fatty acid esters, such as ethyl palmitate or stearate, with acetone in the presence of sodium hydride were unsuccessful in our hands; free fatty acid was usually isolated instead of the expected diketone. Swamer and Hauser (10), however, have reported the preparation of 2,4-pentadecanedione, as well as other β-diketones, by this latter method. They carried out the reaction in a ball mill, whereas we employed conventional glass apparatus with magnetic stirring.

Gas liquid chromatographic retention time data for 2,4-diketones are presented in Table I. It was convenient to express the retention times of the homologues with respect to 2,4-heptadecanedione. Over the range of chain lengths investigated, the plot of log (retention time) vs. number of carbon atoms in the chain was linear for the saturated compounds. On UCW98, Δ^{12}-2,4-heneicosenedione eluted just before

TABLE I

Relative Retention Time Data for 2,4-Diketones

Diketone chain length and type	4% Carbowax on Diatoport S, 6 ft x 1/8 in. SS (He 30 ml/min)		10% UCW98 on Diatoport S, 6 ft x 1/8 in. SS (He 30 ml/min)		
	T 210 C	T 220 C	T 200 C	T 230 C	T 240 C
C_7:0	0.50		0.029		
C_9:0	0.89		0.054		
C_{10}:0	0.123		0.082		
C_{12}:0	0.225		0.166		
C_{13}:0		0.311		0.299	0.322
C_{14}:0		0.414		0.407	0.430
C_{15}:0				0.550	
C_{17}:0	1.00[a]	1.00[b]	1.00[c]	1.00[d]	1.00
C_{18}:0				1.36	
C_{19}:0		1.80		1.85	1.84
C_{20}:0		2.39		2.50	2.36
C_{21}:1		3.35		3.08	
C_{21}:0		3.18		3.40	3.14
C_{23}:0		5.52		6.25	5.59
C_{25}:0				11.50	

[a]Retention time, 5.42 min.
[b]Retention time, 3.70 min.
[c]Retention time, 21.55 min.
[d]Retention time, 4.75 min.

TABLE II

Relative Retention Times of 2,4-Diketone Methoximes and 2,4-Dinitrophenylsemicarbazones

Compound	Relative retention time *of methoxime* Column: 3% OV-17 Temperature: 230 C	Relative retention time of 2,4-dinitrophenyl *semicarbazone* Column: 3% OV-1 Temperature: 200 C
2,4-Tridecanedione	0.153	0.130
2,4-Heptadecanedione	0.535	0.493
2,4-Nonadecanedione	1.00[a]	1.00[b]
2,4-Heneicosanedione	1.84	2.03
2,4-Tricosanedione	3.43	

[a]Retention time, 3,74 min
[b]Retention time, 7.60 min

2,4-heneicosanedione, whereas, on Carbowax 20M, the relative order was reversed, in accordance with their expected behavior. Retention time data for the methoxime and 2,4-dinitrophenylsemicarbazone derivatives of several 2,4-diketones are summarized in Table II. Derivatization with methoxyamine hydrochloride in pyridine has been employed by us in the confirmatory identification of 2,4-diketones obtained by preparative GLC of ketonic material from tissue extracts.

Because of their tautomeric structure, 2,4-diketones undergo silylation with BSA-pyridine or TBT. The trimethylsilyl derivatives may be resolved on columns containing UCW98 stationary phase. These derivatives have not, however, been employed extensively in our investigations.

Infrared absorption spectra of several 2,4-diketones, obtained with liquid films, showed strong absorption at 2940-2780 cm^{-1} and at 1580-1600 cm^{-1}; this latter absorption band is characteristic of the β-diketone structure (22). In the ultraviolet, β-diketones absorb strongly in the region of 270 nm ($n \rightarrow \pi^*$ transition). Because of enolization of active hydrogens, the absorbance is much greater than the summation of the contributions of two simple C=O groups (22). Thus, the observed molar absorption coefficient of 2,4-heptadecanedione in 2,2,4-trimethylpentane at 271 nm was 11,000 ($E^{1\%}_{1cm}$ = 408). The absorption maximum at 273.5 nm

TABLE III

Melting Point Data for 2,4-Diketones

Compound	Melting point °C	Eutectic with Azobenzene °C	Reported melting point	Reference
2,4-Tridecanedione	21.1	–	24-27	5
2,4-Tetradecanedione	26.3	–	28	7
2,4-Pentadecanedione	32.0	–	31-32	7
			31.5-32	9
2,4-Heptadecanedione	42.5	35.0	39	7
2,4-Octadecanedione[a]	46.5	38.5	42	7
2,4-Nonadecanedione[a]	50.5	41.0	49	7
2,4-Eicosanedione[a]	53.7	44.0	51	7
2,4-Heneicosanedione[a]	58.0 59	46.5	52.5	7
2,4-Tricosanedione[a]	62.5	49.7	57	7
2,4-Pentacosanedione[a]	66.8	53.7	–	–

[a]Purified by preparative gas liquid chromatography (GLC) on 3% OV-1.

for 2,4-diketones in 95% ethanol was shifted to 295 nm on the addition of sodium methoxide or sodium hydroxide, with enhanced absorbance at the latter wavelength.

Melting points of several 2,4-diketones, and their eutectic melting points with azobenzene, are summarized in Table III, along with previously reported values. Samples of the diketones were conveniently purified from traces of lower homologues and other impurities by preparative GLC on 3% OV-1.

Mass spectra of 2,4-heptadecanedione, 2,4-nonadecanedione, 2,4-heneicosanedione, Δ^{12}-2,4-heneicosenedione, and 2,4-pentacosanedione gave molecular weights (m/e of parent ion peaks) of 268, 296, 324, 322, and 380, in accord with the calculated values. In all of these mass spectra, a prominent peak at m/e 100 occurred; this peak has been attributed to the ion $C_5H_8O_2^+$, formed by β-elimination of the long alkyl chain by a McLafferty rearrangement (23).

Periodate-permanganate oxidation of saturated 2,4-diketones yielded the expected monocarboxylic acids containing three carbon atoms less than the diketones. Δ^{12}-2,4-Heneicosenedione was cleaved also at the double bond, the final products being nonanedioic and nonanoic acids. The products of alkaline hydrolysis of a 2,4-diketone are acetone and the expected monocarboxylic acid:

$$RCOCH_2COCH_3 \xrightarrow{KOH} RCOOH + CH_3COCH_3$$

The ease with which acetone could be identified by headspace analysis rendered this reaction a valuable confirmatory test for 2,4-diketones when it was applied to preparative GLC fractions from tissue extracts.

The 2,4-diketones are known compounds; however, they have not hitherto been identified as natural products. Since they occur in biological materials as complex mixtures of homologues, it was necessary to isolate them as a group from tissue lipids, to resolve this fraction into individual components, and to identify the latter by physical and chemical techniques and by comparison with authentic 2,4-diketones, the synthesis and properties of which are described in this communication.

The ability of this group of compounds to form chelates with di- and trivalent metal ions, including Ca^{++} and Mg^{++}, is well known. Some of the lower homologues, especially 2,4-pentanedione, and their fluorinated analogues have been employed as analytical reagents because of this property. The metal chelates so formed have the properties of organic compounds; they are soluble in organic solvents such as chloroform and methylene chloride, and some of lower molecular weight may be sublimed. The 2,4-diketones also react readily with compounds possessing a primary amino group to form Schiff bases, and, under appropriate conditions, they may react with -SH as do other active carbonyl compounds. Their widespread distribution in mammalian tissues suggests that they may have some important function or functions in the life process quite apart from their antihistamine activity. Further investigation of these compounds could result in useful therapeutic applications.

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Role and Mechanism of Peripheral Fatty Acid Mobilization in 2-Mercaptoethanol-induced Fatty Liver

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ABSTRACT

2-Mercaptoethanol-induced fatty liver involves an increased free fatty acid mobilization which is primarily due to an inhibition of free fatty acid reesterification in adipose tissue. Furthermore, increased free fatty acid mobilization as well as fatty liver induction are not induced by 2-mercaptoethanol per se but result most probably from 2-mercaptoacetate through oxidation of 2-mercaptoethanol.

INTRODUCTION

We have shown in a recent report (1) that the administration of either 2-mercaptoethanol or 2-mercaptoacetate induces in the rat a fatty liver and a marked and early increase in free fatty acids of blood. These effects, which were not observed after the administration of 2-mercaptopropionate, suggested that increased peripheral fat mobilization could be at least one of the mechanisms involved in the 2-mercaptoethanol and 2-mercaptoacetate induced fatty liver.

In order to test this hypothesis, we have presently investigated the effects of 2-mercaptoethanol and 2-mercaptoacetate on the lipolytic activity of rat adipose tissue. Because 2-mercaptoethanol can lead in vitro to 2-mercaptoacetate through the combined effects of alcohol dehydrogenase and aldehyde dehydrogenase (2), the part played by these thiol-bearing compounds in the stimulation of free fatty acid mobilization and in the induction of fatty liver was also investigated. Fatty acid mobilization is mainly related to an inhibition of free fatty acid reesterification in adipose tissue and is due to 2-mercaptoacetate rather than to 2-mercaptoethanol per se.

MATERIAL AND METHODS

Female Wistar rats, weighing 200 ± 10 g, were fasted overnight preceding the experiments. In the in vivo studies, 2-mercaptoethanol or 2-mercaptoacetate (40 μmol/100 g body weight) were administered intraperitoneally as previously described (1), and control rats received an equal volume of saline. In some experiments, inhibition of alcohol dehydrogenase was induced (3) by an intraperitoneal injection of pyrazole (27 mg/100 g body weight) 10 min before the administration of 2-mercaptoethanol or 2-mercaptoacetate. Blood free fatty acids and liver triacylglycerol were determined as previously described (1). Experiments were also performed to determine (4) the maximal thiol level occurring in the blood after 2-mercaptoethanol and 2-mercaptoacetate administration. In both cases, the maximal blood thiol level occurred 1 hr after the treatment and reached about 2 mM/l. A concentration of 2 mM was therefore chosen in the following studies.

In vitro experiments were performed on parametrial adipose tissue removed from rats fasted overnight. Incubations were for 1 hr, except in the experiments in which pyrazole was used. In the latter, adipose tissue was first preincubated for 30 min in the presence of pyrazole (1 mM), after which 2-mercaptoethanol or 2-mercaptoacetate was added and the fat pads further incubated for an additional hour. Conditions of incubation and of determination of glycerol and free fatty acids released into the medium were those described previously (5). In some experiments, the ATP level in adipose tissue was also determined immediately at the end of the incubation, according to a modification (6) of the enzymatic method of Denton et al. (7). All results are given as the mean ± SEM and Student's *t* test was used to determine statistical significance.

2-Mercaptoethanol and 2-mercaptoacetate were purchased from Merck (Darmstade, Germany), pyrazole from Fluka (Buchs, Switzerland), bovine albumin (Pentex, fraction V, fatty acid poor) from Miles Labs (Kankakee, IL). The enzymatic determinations were carried out with reagents from Boehringer (Mannheim, Germany). All other chemicals were of analytical grade.

RESULTS

The influence of 2-mercaptoethanol and

TABLE I

In Vitro Effects of 2-Mercaptoethanol and 2-Mercaptoacetate on the Basal Lipolytic Activity and on the ATP Content of Parametrial Adipose Tissue from Fasted Rats[a]

	Addition to the medium		
	O	2-Mercaptoethanol (2.10^{-3}M)	2-Mercaptoacetate (2.10^{-3}M)
Glycerol release (μmol/g wet wt/hr)	1.88 ± 0.29 (5)	2.14 ± 0.44 (5) $P > 0.05$	2.53 ± 0.40 (5) $0.001 < P < 0.01$
Free fatty acid release (μ mol/g wet wt/hr)	2.95 ± 0.50 (5)	6.23 ± 0.93 (5) $0.001 < P < 0.01$	7.08 ± 1.96 (5) $P < 0.001$
ATP level (nmol/g wet wt)	227 ± 75 (6)	103 ± 34 (7) $0.001 < P < 0.01$	90 ± 31 (9) $0.001 < P < 0.01$

[a]Parametrial adipose tissue was incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing glucose (5 mM) and albumin (1%, w/v). Glycerol and free fatty acids released into the medium and adipose tissue ATP level were determined after 1 hr incubation in the presence or absence of 2-mercaptoethanol or 2-mercaptoacetate. Each value represents the mean ± SEM with the number of determinations in parentheses.

2-mercaptoacetate (2 mM) on the basal lipolytic activity of parametrial adipose tissue from fasted rats is shown in Table I. The rate of free fatty acid release was markedly enhanced by both compounds (2.1- and 2.4-fold, respectively). In contrast, the rate of glycerol release was unaffected by 2-mercaptoethanol and significantly increased by 2-mercaptoacetate. Although the rate of esterification of free fatty acid was not presently investigated, these results suggested that 2-mercaptoethanol and 2-mercaptoacetate may reduce the free fatty acid reesterification process in adipose tissue and led us to study the influence of these compounds on adipose tissue ATP level. As shown in Table I, the ATP level in adipose tissue incubated in the presence of 2-mercaptoethanol or 2-mercaptoacetate was severely depressed (54 and 60%, respectively).

The influence of inhibition of alcohol dehydrogenase on the marked increase in blood free fatty acid level which follows the administration of 2-mercaptoethanol or 2-mercaptoacetate is shown in Table II. When the alcohol dehydrogenase inhibitor, pyrazole (3), was injected prior to 2-mercaptoethanol or 2-mercaptoacetate, the rise in blood free fatty acids occurring 3 or 8 hr after the treatment was almost completely prevented in the case of 2-mercaptoethanol, whereas it was unaltered in the case of 2-mercaptoacetate. Furthermore, pyrazole influenced in a similar fashion the liver triacylglycerol accumulation induced by 2-mercaptoethanol or 2-mercaptoacetate (Table II). In fact, pyrazole reduced or completely abolished the increase in liver triacylglycerol found 3 or 8 hr after 2-mercaptoethanol administration, whereas, under the same conditions, it failed to affect significantly the magnitude of the 2-mercaptoacetate-induced fatty liver.

In order to determine whether pyrazole may also inhibit in vitro the stimulating effect of 2-mercaptoethanol on free fatty acid release from adipose tissue, lipolysis was studied in fat pads first exposed to pyrazole (1 mM) and then incubated in the presence of 2-mercaptoethanol (2 mM). As shown in Table III, preincubation with pyrazole resulted in the suppression of the stimulating effect of 2-mercaptoethanol on free fatty acid release. Under the same conditions, however, preincubation with pyrazole failed to alter the effects of 2-mercaptoacetate.

DISCUSSION

The present studies show that the fatty liver induced by 2-mercaptoethanol or by 2-mercaptoacetate is accompanied by a large increase in the blood free fatty acids, an increase which seems related to a stimulatory action of these thiol-bearing compounds on the rate of free fatty acid mobilization from adipose tissue. Furthermore, this stimulatory effect appears to result from a decreased rate of free fatty acid reesterification in adipose tissue rather than from stimulated lipolysis, as shown by the fall in adipose tissue ATP level and the large increase in the free fatty acid output compared with the slight modification in the glycerol released. These effects, which were observed under basal conditions, contrast with the marked antilipolytic action of 2-mercaptoethanol against epinephrine-induced lipolysis first shown by Lavis et al. (8) and confirmed during the course of these experiments (data not shown).

On the other hand, the present data also indicate that pyrazole abolishes the ability of

TABLE II

Influence of Pyrazole on the Hepatic Triacylglycerol and the Blood Free Fatty Acid Levels in the Rat, 3 and 8 hr after the Intraperitoneal Administration of 2-Mercaptoethanol or 2-Mercaptoacetate (40 μmol/100 g body weight)[a]

Time after treatment	3 Hr		8 Hr	
	Hepatic triacylglycerol (mg/g wet wt)	Blood free fatty acids (μequiv./l)	Hepatic triacylglycerol (mg/g wet wt)	Blood free fatty acids (μequiv./l)
Control	6.0 ± 1.0 (5)	829 ± 141 (5)	5.3 ± 1.4 (7)	713 ± 157 (5)
2-Mercaptoethanol	17.2 ± 6.2 (3) 0.001 < P < 0.01 (a)	2904 ± 212 (3) P < 0.001 (a)	19.0 ± 4.3 (3) P < 0.001 (a)	1155 ± 201 (3) 0.01 < P < 0.05 (a)
2-Mercaptoacetate	17.3 ± 4.4 (3) 0.001 < P < 0.01 (a)	3465 ± 220 (3) P < 0.001 (a)	47.7 ± 6.1 (3) P < 0.001 (a)	1373 ± 316 (3) 0.001 < P < 0.01 (a)
Pyrazole	5.2 ± 2.1 (3) P > 0.05 (a)	759 ± 87 (3) P > 0.05 (a)	7.8 ± 1.2 (6) P > 0.05 (a)	801 ± 134 (6) P > 0.05 (a)
2-Mercaptoethanol + Pyrazole	10.4 ± 2.5 (5) 0.01 < P < 0.05 (a,b)	992 ± 91 (5) P > 0.05 (a,b)	5.6 ± 0.4 (5) P > 0.05 (a,b)	749 ± 160 (5) P > 0.05 (a,b)
2-Mercaptoacetate + Pyrazole	15.4 ± 4.3 (5) 0.001 < P < 0.01 (a,b)	2543 ± 180 (5) P < 0.001 (a,b)	44.2 ± 7.5 (5) P < 0.001 (a,b)	1530 ± 89 (5) P < 0.001 (a,b)

[a]Pyrazole (27 mg/100 g body weight) was injected intraperitoneally 10 min before the administration of 2-mercaptoethanol or 2-mercaptoacetate. Each value represents the mean ± SEM with the number of determinations in parentheses. Statistical significance: (a) compared with control, (b) compared with pyrazole.

TABLE III

Influence of Pyrazole on the Effects of 2-Mercaptoethanol and 2-Mercaptoacetate on the Basal Lipolytic Activity of Parametrial Adipose Tissue from Fasted Rats[a]

	Addition to the medium			
	O	Pyrazole (1.10^{-3}M)	Pyrazole (1.10^{-3}M)+ 2-Mercaptoethanol (2.10^{-3}M)	Pyrazole (1.10^{-3}M) + 2-Mercaptoacetate (2.10^{-3}M)
Glycerol release (μmol/g wet wt/hr)	1.73 ± 0.19 (8)	1.70 ± 0.30 (10) P > 0.05 (a)	2.39 ± 0.77 (5) P > 0.05 (a,b)	2.70 ± 0.37 (5) 0.001 < P < 0.01 (a,b)
Free fatty acid release (μmol/g wet wt/hr)	2.93 ± 0.57 (8)	2.58 ± 0.39 (8) P > 0.05 (a)	2.22 ± 0.97 (5) P > 0.05 (a,b)	5.54 ± 0.66 (5) P < 0.001 (a,b)

[a]Parametrial adipose tissue was incubated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing glucose (5 mM) and albumin (1%, w/v). After a 30 min preincubation period in the presence or absence of pyrazole (10^{-3}M), the glycerol and free fatty acid release was determined. 2-Mercaptoethanol or 2-mercaptoacetate (2.10^{-3}M) was then added and the adipose tissue further incubated for an additional hour. The glycerol and free fatty acid released during the first 30 min preincubation was subtracted from the release determined at the end of the final incubation. Each value represents the mean ± SEM with the number of determinations in parentheses. Statistical significance: (a) compared with control, (b) compared with pyrazole.

2-mercaptoethanol, but not of 2-mercaptoacetate, to induce in vitro an increase in the rate of free fatty acid mobilization from adipose tissue and in vivo both a fatty liver and an increase in blood free fatty acids. Because pyrazole is a potent inhibitor of alcohol dehydrogenase (3,9,10) and because we have shown that this compound in the dosage used presently does not interfere with lipolysis both in vitro and in vivo (11,12), the following conclusions are made:

The increase in free fatty acid mobilization from adipose tissue and the fatty liver induced by 2-mercaptoethanol are due to 2-mercaptoacetate or to one of its metabolites rather than to 2-mercaptoethanol per se.

2-Mercaptoethanol, which has been previously described as a substrate for rat liver alcohol dehydrogenase in vitro (2), appears to be also oxidized in vivo by this enzyme.

Although alcohol dehydrogenase activity in adipose tissue is low (13) in comparison with liver (13), the oxidation of 2-mercaptoethanol in adipose tissue in vitro seems also to involve this enzyme, as suggested by the inhibitory action of pyrazole. Previous results concerning the relative lipolytic activity of ethanol (14) and acetaldehyde (5,15) in rat adipose tissue in vitro favor also the existence of a functional alcohol dehydrogenase in this tissue.

Increased free fatty acid mobilization appears to be one, if not the major, mechanism involved in the pathogenesis of the 2-mercaptoethanol- and of the 2-mercaptoacetate-induced fatty liver.

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Sterol Mutants of *Saccharomyces cerevisiae*: Chromatographic Analyses

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ABSTRACT

The sterols accumulated by ergosterol deficient mutants of the genes *erg6, erg2, erg3,* and *erg5* (formerly *pol1, pol2, pol3,* and *pol5*) have been analyzed by gas liquid chromatography. Together with pure sterols obtained from the mutants, they were characterized on SE-30, OV-17, and OV-225. The effects of molecular structure on the retention characteristics of a range of C_{28} ergostane sterols have been studied. The double mutants obtained by crossing the single mutants were also analyzed and their sterols identified where possible. The effects of the *erg* mutations on the control of sterol biosynthesis in yeast are discussed.

INTRODUCTION

Auxotrophic mutants have been widely used in the analysis of anabolic pathways in microorganisms, particularly *Escherichia coli* and *Neurospora crassa* (1,2). The rationale behind the procedures and techniques employed in such studies is documented in the above texts. Attempts have been made to isolate sterol-requiring mutants of yeast (*Saccharomyces cerevisiae*) on the assumption that ergosterol, the end product of sterol biosynthesis in yeast, is essential for growth (3-5). Of these, the latter two investigations were successful. In addition, several other investigators have reported mutants which respond to ergosterol or an unsaturated fatty acid (6,7) or ergosterol plus an unsaturated fatty acid (8). The former mutants were termed *ole* and the latter *olerg*. However, the *ole2, 3,* and *4* mutants have been shown to be primarily defective in porphyrin biosynthesis and cannot complete ergosterol biosynthesis due to the absence of a heme cofactor (9). The *olerg* mutants also lack or are defective in porphyrin biosynthesis and cannot complete ergosterol biosynthesis due to the absence of a heme cofactor (9). The *olerg* mutants also lack or are defective in porphyrins (Woods and Gardner, unpublished results) and the *erg*- mutants (5) respond to porphyrin supplements. Thus, only the mutant, *erg1,* (4) appears to be a true sterol mutant.

An alternative approach to the isolation of sterol mutants has been to select for resistance to polyene antibiotics such as nystatin. Polyenes act by complexing with sterols in the cell membrane, thus causing loss of selective permeability and cell death (10). Altered sterol composition in polyene resistant mutants has been reported in *Saccharomyces cerevisiae* (7,11,12), *Candida albicans* (13), *Candida tropicalis* (14), and *Neurospora crassa* (15). Genetic analysis of the mutants of *S. cerevisiae* resulted in the characterization of four genes, *pol1, pol2, pol3,* and *pol5* which gave altered sterol patterns (12). None of these mutants was auxotrophic for sterols.

The biosynthesis of ergosterol in yeast has been investigated in a number of laboratories (16-18). These investigations have all used the incorporation of radioactively labeled precursors of ergosterol to deduce the operative sequence of the pathway. Such studies lead to the conclusion that there is no specific route to ergosterol; ergosterol can be formed by any one of several tracks through a complex network of reactions (17,18).

The results of ultraviolet spectrophotometry and the Liebermann-Burchard test on sterols extracted from the *pol* mutants (12) suggested that they were defective in the terminal steps of the pathway; namely the conversion of zymosterol (cholesta-8,24-dien-3β-ol) to ergosterol (ergosta-5,7,22-trien-3β-ol). This conversion in-

TABLE I

Presumptive Enzyme Defects in the *erg* Mutants

Mutant designation		
Present	Previous	Biochemical lesion
erg 6	*pol 1, nys 1*	C-24 transmethylation
erg 2	*pol 2*	$\Delta^{8}\rightarrow\Delta^{7}$ isomerization
erg 3	*pol 3, nys 3*	5(6) desaturation
erg 5	*pol 5*	22(23) desaturation

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TABLE II

Standard Sterols: Nomenclature

Chemical name	Trivial name	Common name
Cholesta-8,24,-dien-3β-ol	$\Delta^{8,24}_{C}$	zymosterol
Cholesta-5,7,24-trien-3β-ol	$\Delta^{5,7,24}_{C}$	---
Cholesta-5,7,22,24-trien-3β-ol	$\Delta^{5,7,22,24}_{C}$	---
Ergosta-8-3en-3β-ol	Δ^{8}_{E}	---
Ergosta-8,24(28)-dien-3β-ol	$\Delta^{8,24(28)}_{E}$	fecosterol
Ergosta-8,22,-dien-3β-ol	$\Delta^{8,22}_{E}$	---
Ergosta-8,22,24(28)-trien-3β-ol	$\Delta^{8,22,24(28)}_{E}$	---
Ergosta-7-en-3β-ol	Δ^{7}_{E}	---
Ergosta-7,24(28)-dien-3β-ol	$\Delta^{7,24(28)}_{E}$	episterol
Ergosta-7,22-dien-3β-ol	$\Delta^{7,22}_{E}$	---
Ergosta-7,22-24,(28)-trien-3β-ol	$\Delta^{7,22,24(28)}_{E}$	---
Ergosta-5,8,22-trien-3β-ol	$\Delta^{5,8,22}_{E}$	—
Ergosta-5,7-dien-3β-ol	$\Delta^{5,7}_{E}$	---
Ergosta-5,7,22-trien-3β-ol	$\Delta^{5,7,22}_{E}$	ergosterol
Ergosta-5,7,22-24(28)-tetraen-3β-ol	$\Delta^{5,7,22,24(28)}_{E}$	24(28)dehydroergosterol

volves the following molecular changes:

(a) transmethylation at C-24 to give a 24(28) double bond
(b) isomerization of Δ^8 to Δ^7
(c) desaturation at 5(6)
(d) desaturation at 22(23)
(e) reduction of the 24(28) double bond

These rearrangements are shown in Figure 1.

Sterols accumulated by the *pol* mutants and their parent wild type strain have been identified by Barton et al. (19,20) as well as analysis of the sterols in the double mutants (21). We report here an analysis by gas liquid chromatography (GLC) of the sterols in both the single and double mutants. Our results both confirm but also extend the results of the chemical analyses. In addition, (a) we compare the sterol composition of sterol mutants grown under both fermentative and oxidative conditions and (b) demonstrate that double bond alterations in the sterol nucleus and sterol side chain result in various discrete separation factors on nonpolar and polar GLC columns. The latter analysis will enable investigators to make certain predictions regarding the relative retention times of similar sterols in the absence of sterols standards. In conclusion, various aspects of yeast ergosterol biosynthesis based upon the accumulated sterols in the mutants are discussed.

To reduce possible nomenclatural confusion between these mutants which are defective in sterol biosynthesis and nucleic acid polymerase mutants in yeast, we have redesignated the *pol* mutants as *erg* – for ergosterol-deficient. Thus, *pol1, 2, 3,* and *5* become *erg6, erg2, erg3,* and *erg5,* respectively. These changes are listed in Table I. *Erg4,* a mutant unable to reduce the 24(28) double bond, will be reported elsewhere.

For convenience in referring to the complex mixtures of cholestanes and ergostanes present in the mutants, we have adopted the following notation:

cholestanes Δ_C, ergostanes Δ_E.

Thus, zymosterol – Cholesta-8,24-dien-3β-ol – is written $\Delta^{8,24}_{C}$, and fecosterol – ergosta-8,24(28)-dien-3β-ol – is written $\Delta^{8,24(28)}_{E}$. A full list of sterols used together with their chemical, common, and trivial nomenclature is given in Table II.

METHODS AND MATERIALS

Yeast Strains

The provenance of the wild type strain A184D (erg^+) and the mutants of *erg6, 2, 3,* and *5,* has been described previously (12). The original *erg3* mutant contained trace amounts of ergosterol; in this work we used another *erg3* allele, SM36, which contains no ergosterol. The double mutants *erg6/2, erg6/3, erg6/5, erg2/3, erg2/5,* and *erg3/5* were synthesized by conven-

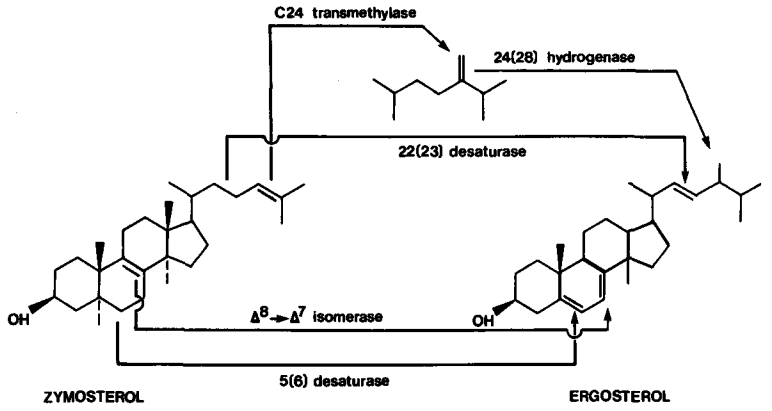


FIG. 1. Double bond changes necessary in the nucleus and side chain to convert zymosterol to ergosterol.

tional genetic techniques and characterized by UV spectrophotometry of extracted sterols (see Results). Petite derivatives were induced by overnight incubation in yeast complete medium + 15 μg/ml ethidium bromide.

Media

Yeast complete medium (YCM) contained yeast extract (0.5%), casein hydrolyzate (0.5%), peptone (0.3%), and was at pH 5.8. The carbon source was glucose, 4%, or ethanol, 1%.

Growth Conditions

Cultures were grown in liquid YCM for 48 hr at 28 C from an initial inoculum of 10^6 cells/ml on a rotary shaker at 200 rpm. The culture vessels were Erlenmeyer flasks containing one-fifth of their normal capacity of medium.

Sterol Extractions

Nonsaponifiable sterols from ca. 1 g wet weight of cells were extracted into n-heptane (10 ml) by the method of Breivik and Owades (22). They were checked routinely by UV spectrophotometry before further analysis.

Gas Liquid Chromatography

Nonsaponifiable extracts were evaporated to dryness under vacuum and acetylated with acetic anhydride-pyridine (5:1). The excess acetylating reagents were removed by a stream of nitrogen. In some experiments, the extracts were not acetylated but analyzed as free sterols.

Extracts were dissolved in acetone and analyzed in a Pye 104 chromatograph on the following columns:

(a) SE-30, 1% on Gas Chrom Q (80-100 mesh) 7 ft glass column at 236 C.
(b) OV-17, 1% on Diatomite CLQ (100-120 mesh) 9 ft glass column at 271 C.
(c) OV-225, 3% on Gas Chrom Q (100-120 mesh) 7 ft glass column at 256 C.

The carrier gas was argon at 60 ml/min for all columns. Retention times of sterol acetates were calculated relative to cholestane (SE-30), and cholestanol acetate (OV-17), OV-225). The standard for free sterols on SE-30 was cholesterol. Reproducibility in relative retention times (RRT) was SE-30 ± 3%, OV-17 ± 1.5%, OV-225 ± 1.5%.

Standard Sterols

These were isolated and identified by Corrie (20) and are listed in Table III.

Materials

Spectroscopic grade n-heptane (Fluka AG) was obtained from Fluorochem Ltd., Glossop, England. Gas Chrom Q coated with SE-30 and OV-225 were purchased from Phase-Sep Ltd., Queensferry, Flintshire, N. Wales, and Diatomite coated with OV-17 from J.J.'s Chromatography Ltd., Kings Lynn, Norfolk, England. All other reagents and chemicals were Analar grade.

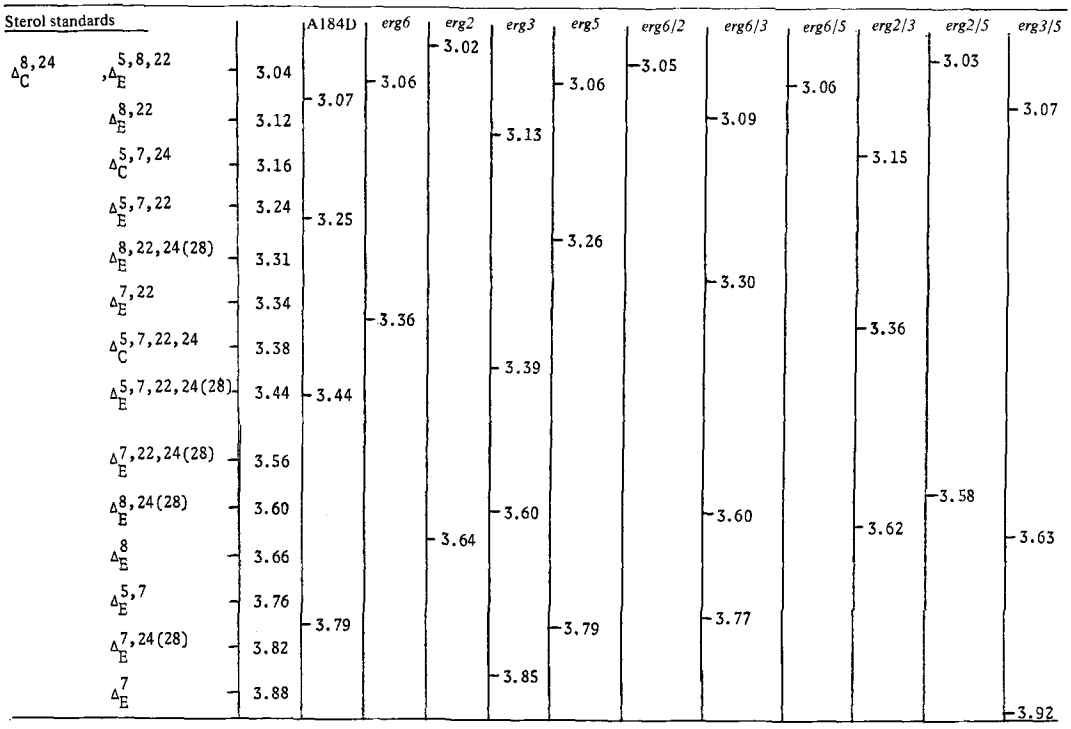
RESULTS

Sterol Standards

Several workers have investigated the effects of molecular structure on the GLC characteristics of sterols (23,24) and have shown that the results have predictive value. We have carried out a similar analysis with the range of C_{28} (ergostane) sterols obtained from the *erg* mutants. The results are listed in Table VI. It can be seen that the separation factors for both nucleus and side chain alterations are constant for the ergostanes. The results obtained on SE-30 agree closely with those of Patterson (24).

TABLE III

Relative Retention Times of Sterol Standards and Sterol from the Single and Double Mutants on a 1% SE-30 Column



Single Mutants

The identification of the sterols present in the mutants has to take into account the data from ultraviolet spectrophotometry (12). This indicated the following: (a) A184D contains ergosterol ($\Delta^{5,7,22}_{E}$) and 24 (28)-dehydroergosterol ($\Delta^{5,7,22,24(28)}_{E}$), (b) *erg6* contains both $\Delta^{5,7}_{E}$ and side chain dienes (UV max 230-235 nm), (c) *erg2* has no diene containing sterols, (d) *erg3* has sterols with a side chain diene (UV max 225-230 nm), and (e) *erg5* has sterols with a $\Delta^{5,7}_{E}$ diene system.

The sterol identifications obtained by correlating the above structural requirements with the retention data of sterol extracts on all three columns are listed in Tables III-V.

Double Mutants

Here again, the results of the spectrophotometric analysis place restrictions on the structures of possible sterols. Four of the mutants, *erg6/2, erg2/3, erg2/5,* and *erg3/5,* contain sterols which lack diene systems; in *erg6/3* there is a sterol with a side chain diene (UV max 230-235 nm) which, by analogy with the single mutants, can be deduced to be $\Delta^{22,24}$, and in *erg6/5,* there is a $\Delta^{5,7}$ diene sterol. In addition, the biochemical lesions (see Table I) place further restrictions on the possible sterols. Tables III-V list the sterols with their relative retention times as detected on all three GLC columns.

Effects of Oxidative and Fermentative Metabolism on Sterol Composition

Since the sterol composition of the wild type strains was known to be affected by cultural conditions (25), it was decided to study the mutants under similar conditions. Wild type and the single mutants were compared under the following conditions: (a) YCM, (b) YCM with ethanol replacing glucose as carbon source and (c) the petite derivatives on YCM. The sterols were extracted and analyzed as free sterols on SE-30. The chromatograms were reproducible and the resolution obtained was not significantly different from that found with sterol acetates.

The results for wild type indicate that ergosterol, zymosterol, and 24(28) dehydroergosterol are accumulated in equivalent quantities in YCM but that, under other conditions, ethanol replacing glucose and the petite derivative of wild type grown on YCM, ergosterol, predominates. Analogous quantitative, rather

TABLE IV

Relative Retention Times of Sterol Standards and Sterols from the Single and Double Mutants on a 1% OV-17 Column

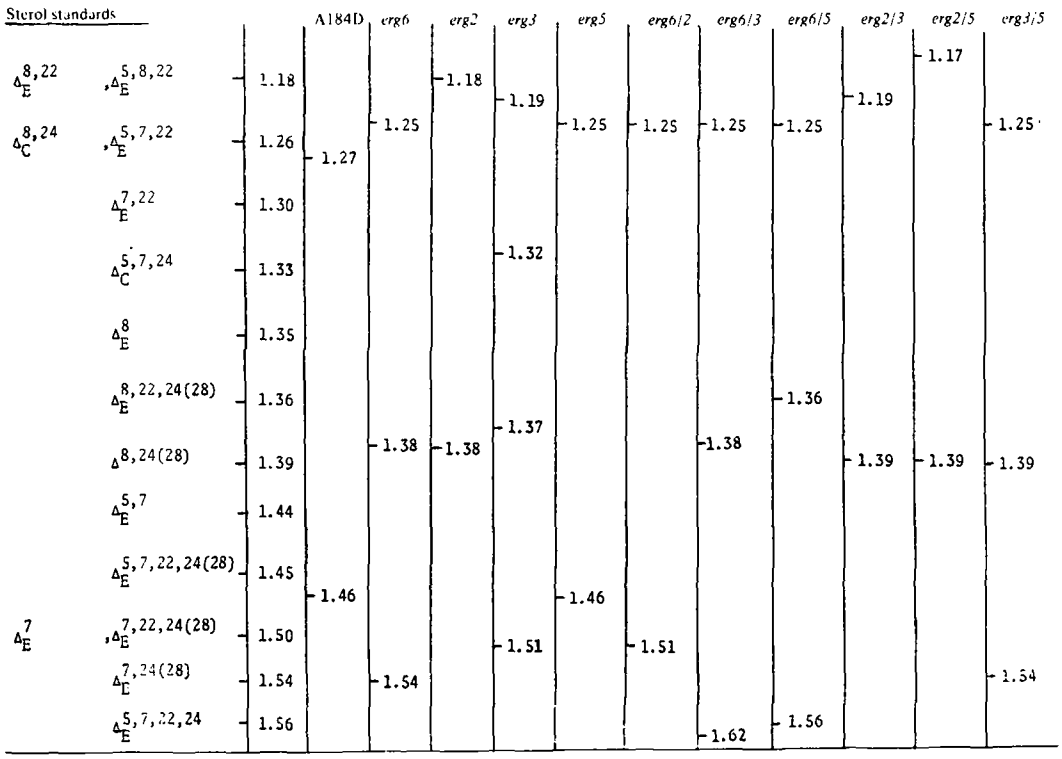


TABLE V

Relative Retention Times of Sterol Standards and Sterols from the Single and Double Mutants on a 3% OV-225 Column

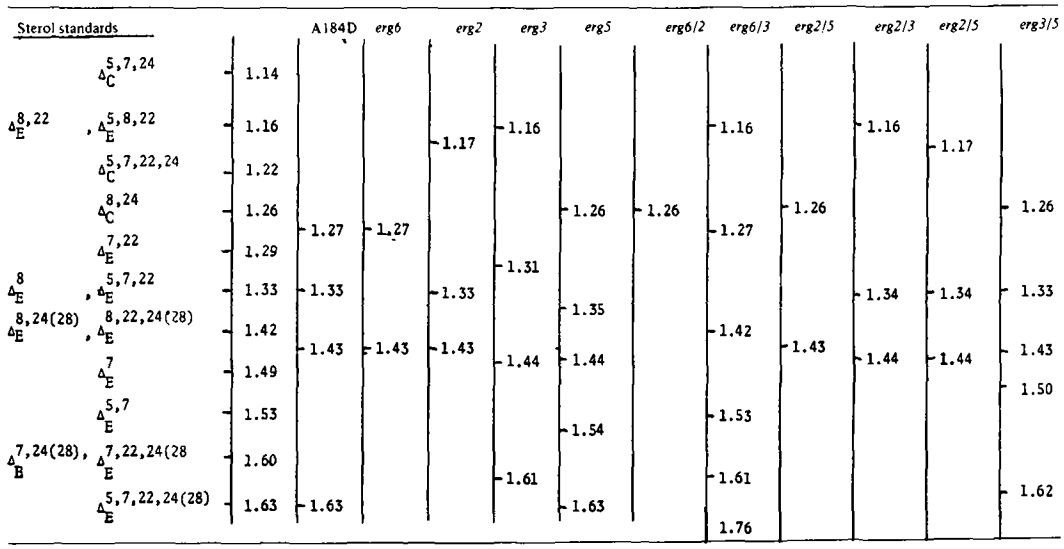


TABLE VI

Effects of Molecular Structure on Sterol Separations

Comparison		Side chain unsaturation	Carbon number	Separation factors SE-30	OV-17	OV-225
A. Nucleus changes						
Ergostanes	Δ^{8}/Δ^{7}	-	28	0.94	0.90	0.89
	Δ^{8}/Δ^{7}	Δ^{22}	28	0.93	0.91	0.90
	Δ^{8}/Δ^{7}	$\Delta^{24(28)}$	28	0.94	0.90	0.89
	Δ^{8}/Δ^{7}	$\Delta^{22,24(28)}$	28	0.93	0.91	0.89
	$\Delta^{8}/\Delta^{5,7}$	-	28	0.97	0.94	0.87
	$\Delta^{8}/\Delta^{5,7}$	Δ^{22}	28	0.96	0.94	0.87
	$\Delta^{8}/\Delta^{5,7}$	$\Delta^{22,24(28)}$	28	0.96	0.94	0.88
	$\Delta^{7}/\Delta^{5,7}$	-	28	1.03	1.04	0.97
	$\Delta^{7}/\Delta^{5,7}$	Δ^{22}	28	1.03	1.03	0.97
	$\Delta^{7}/\Delta^{5,7}$	$\Delta^{22,24(28)}$	28	1.03	1.03	0.98
Cholestanes	$\Delta^{8}/\Delta^{5,7}$	Δ^{24}	27	0.96	0.95	1.10
B. Side chain changes						
Substituent		Comparison				
Ergostanes	Δ^{22}	$\Delta^{8,22}/\Delta^{8}$	28	0.85	0.87	0.87
		$\Delta^{7,22}/\Delta^{7}$	28	0.86	0.87	0.87
		$\Delta^{5,7,22}/\Delta^{5,7}$	28	0.86	0.87	0.87
		$\Delta^{8,22,24(28)}/\Delta^{8,24(28)}$	28	0.92	0.98	1.00
		$\Delta^{7,22,24(28)}/\Delta^{7,24(28)}$	28	0.93	0.97	1.00
	$\Delta^{24(28)}$	$\Delta^{8,24(28)}/\Delta^{8}$	28	0.98	1.03	1.07
		$\Delta^{7,24(28)}/\Delta^{7}$	28	0.98	1.03	1.07
		$\Delta^{8,22,24(28)}/\Delta^{8,22}$	28	1.06	1.15	1.22
		$\Delta^{7,22,24(28)}/\Delta^{7,22}$	28	1.07	1.15	1.24
		$\Delta^{5,7,22,24(28)}/\Delta^{5,7,22}$	28	1.06	1.15	1.22
	$\Delta^{22,24(28)}$	$\Delta^{8,22,24(28)}/\Delta^{8}$	28	0.90	1.01	1.07
		$\Delta^{7,22,24(28)}/\Delta^{7}$	28	0.92	1.00	1.07
		$\Delta^{5,7,22,24(28)}/\Delta^{5,7}$	28	0.91	1.01	1.06
Cholestanes	Δ^{22}	$\Delta^{5,7,22,24}/\Delta^{5,7,24}$	27	1.07	1.17	1.07

than qualitative changes were observed for the mutants. The petite derivatives showed the most marked changes. Thus, *erg6* petite revealed the presence of $\Delta^{5,7,24}_{C}$ which had been demonstrated chemically but not by GLC. In *erg2* petite, the levels of $\Delta^{8,24(28)}_{E}$, $\Delta^{5,8,22}_{E}$, and $\Delta^{8,22}_{E}$ were approximately equal, whereas in the normal strain $\Delta^{824(28)}_{E}$ is the major accumulation. The composition of *erg5* also changes in that a peak with an RRT corresponding to that of $\Delta^{8,24(28)}_{E}$ becomes easily discernible in the petite derivative while it is barely detectable in the normal strain. It was previously reported that the sterol composition of *erg3* was markedly altered in the petite derivative (11). The mutants used in this and other early work (12) were leaky and accumulate some ergosterol; their petite derivatives do

TABLE VII

Sterols of the *erg* Mutants Identified by Chemical and Chromatographic Analysis

Yeast strain	Chemical analysis	Total	GLC analysis	Total
A184D	$\Delta_C^{8,24}$, $\Delta_E^{5,7,22}$	2	$\Delta_C^{8,24}$, $\Delta_E^{8,24(28)}$, $\Delta_E^{7,24(28)}$, $\Delta_E^{5,7,22}$, $\Delta_E^{5,7,22,24(28)}$	5
erg6	$\Delta_C^{8,24}$, $\Delta_C^{5,7,24}$, $\Delta_C^{5,7,22,24}$	3	$\Delta_C^{8,24}$, $\Delta_C^{5,7,22,24}$ + 1	3
erg2	Δ_E^{8}, $\Delta_E^{8,24(28)}$, $\Delta_E^{8,22}$, $\Delta_E^{5,8,22}$	4	Δ_E^{8}, $\Delta_E^{8,24(28)}$, $\Delta_E^{8,22}$ and/or $\Delta_E^{5,8,22}$	3–4
erg3	$\Delta_E^{8,24(28)}$, $\Delta_E^{8,22}$, $\Delta_E^{8,22,24(28)}$, $\Delta_E^{7,24(28)}$, $\Delta_E^{7,22}$, $\Delta_E^{7,22,24(28)}$	6	$\Delta_E^{8,22}$, $\Delta_E^{8,24(28)}$ and/or $\Delta_E^{8,22,24(28)}$, $\Delta_E^{7,22}$, $\Delta_E^{7,24(28)}$ and/or $\Delta_E^{7,22,24(28)}$	4
erg5	$\Delta_E^{7,24(28)}$, $\Delta_E^{5,7}$, $\Delta_E^{5,7,24(28)}$, $\Delta_E^{8,14,24(28)}$	4	$\Delta_C^{8,24}$, $\Delta_E^{7,24(28)}$, $\Delta_E^{5,7}$ + 2	5
erg6/2	$\Delta_C^{8,24}$	1	$\Delta_C^{8,24}$ + 1	2
erg6/3	$\Delta_C^{8,24}$, $\Delta_C^{7,24}$	2	$\Delta_C^{8,24}$ + 5	6
erg6/5	$\Delta_C^{8,24}$, $\Delta_C^{5,7,24}$	2	$\Delta_C^{8,24}$ + 2	3
erg2/3	$\Delta_E^{8,24(28)}$	1	$\Delta_E^{8,24(28)}$, Δ_E^{8}, $\Delta_E^{8,22}$ + 1	4
erg2/5	$\Delta_E^{8,24(28)}$, Δ_E^{8}, $\Delta_E^{5,8}$	3	$\Delta_E^{8,24(28)}$, + 2	3
erg3/5	$\Delta_C^{8,24}$, $\Delta_E^{8,24(28)}$, $\Delta_E^{7,24(28)}$, Δ_E^{7}	4	$\Delta_C^{8,24}$, $\Delta_E^{8,24(28)}$, Δ_E^{8}, $\Delta_E^{7,24(28)}$, Δ_E^{7}	5

not accumulate UV detectable levels of $\Delta_E^{22,24(28)}$ sterols. In contrast, the *erg3* mutant (SM36) a nonleaky mutant obtained from S. Molzahn used in this study contains no ergosterol and accumulates $\Delta_E^{22,24(28)}$ sterols under all conditions tested.

DISCUSSION

The aim of this study was twofold, (a) to characterize the standard sterols obtained from the *erg* mutants by GLC and (b) to provide chromatographic fingerprints of the altered sterol composition of the mutants and, where possible, to correlate these two sets of data. These aims will be considered in turn.

The effects of sterol structure on retention characteristics have been investigated by several workers (23,24). Unfortunately the choice of liquid phases for GLC analysis is generally arbitrary and in any event subject to new developments. The phases used in this study were chosen to cover a maximum range of polarity; SE-30 being the least and OV-225 the most polar. The retention data and the effects of molecular changes on retention times on SE-30 (1%) agree closely with those obtained by Patterson on a 3% column. The comparative

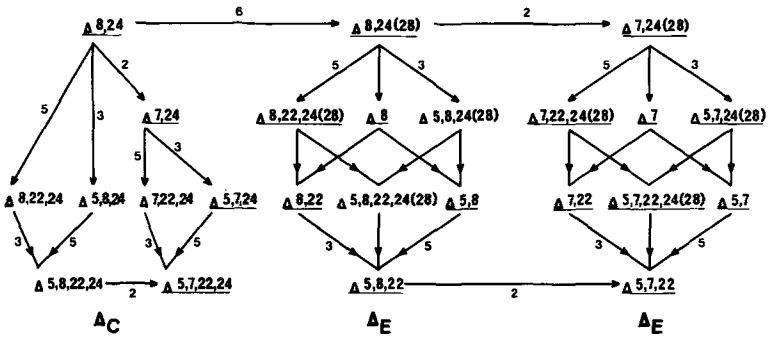


FIG. 2. Theoretical reaction network in sterol biosynthesis based on sterols found in wild type and mutant *Saccharomyces cerevisae*. Sterols actually identified in these strains have been underlines. $\underline{\ }$ indicates that this reaction does not occur in *erg6* or one of the other designated mutants. Sterols above the Δ_C designation belong to the cholestane or 27 carbon atom family of sterols. Similarly, sterols above the Δ_E designation belong to the ergostane or 28-carbon atom family of sterols.

data for the ergostane series are more extensive than that found by Patterson (24) and allow several useful observations to be made. First, the effects of nuclear changes, e.g., Δ^8 vs. Δ^7 on retention times, are independent of the degree of side chain unsaturation (Table VI-A). Second, the effects of individual side chain unsaturation configurations are dependent on the overall composition of the side chain. For example, the change from Δ^8 to $\Delta^{8,22}$ or Δ^7 to $\Delta^{7,22}$ gives a constant separation factor for each of the three columns, whereas the change from $\Delta^{8,24(28)}$ to $\Delta^{8,22,24(28)}$, which also involves the introduction of a Δ^{22}, gives a different separation factor. It is possible to draw up a series of squence relationships for the ergostanes on each column. Thus, on SE-30 sterols with identical side chains chromatograph in the sequence

$$\Delta^8 \rightarrow \Delta^{5,7} \rightarrow \Delta^7$$

while differing side chains on a common nucleus will have the sequence

$$\Delta^{22} \rightarrow \Delta^{22,24(28)} \rightarrow \Delta^{24(28)} \rightarrow \Delta^0$$

where Δ^0 indicates a fully saturated side chain. On OV-17, the sequences were

$$\Delta^8 \rightarrow \Delta^{5,7} \rightarrow \Delta^7$$

and

$$\Delta^{22} \rightarrow \Delta^O \rightarrow \Delta^{22,24(28)} \rightarrow \Delta^{24(28)}$$

while, on OV-225 they were

$$\Delta^8 \rightarrow \Delta^7 \rightarrow \Delta^{5,7}$$

and

$$\Delta^{22} \rightarrow \Delta^0 = \Delta^{24(28)} \rightarrow \Delta^{22,24(28)}$$

By using three columns, it was possible to characterize and identify the majority of the sterols accumulated by both single and double mutants. The results of the chromatographic and chemical analyses (19,21) are compared in Table VII. The results are in close agreement for the single mutants, less so for the double mutants. Most of the differences can be attributed to the different growth conditions in the two laboratories and to the greater sensitivity of GLC to minor components of the sterol mixture. The resolving powers of chemical analysis and GLC were complementary in that the former was able to separate sterols differing only in side chain unsaturation, e.g., $\Delta^{8,24(28)}$ and $\Delta^{8,22,24(28)}$ in *erg3* (which was not always possible by GLC), whereas the latter technique separated sterols with identical side chains but different nuclei. The chromatograms of both single and double mutants were sufficiently distinctive to be used for identifying the genetic constitution of segregants obtained by meiotic analysis.

The data in Table VII allow the formulation of some useful and interesting observations on the biosynthetic pathway from zymosterol to ergosterol. It has been apparent for some time that there is no single route for the synthesis of ergosterol (17,18). The availability of mutants defective in four of the five necessary enzymic steps and the characterization of the sterols accumulated by these mutants make it possible to draw up a more meaningful reaction network than has been possible hitherto. This network is shown in Figure 2. Sterols which have been detected in the wild type and mutant strains are underlined.

Considering the mutants in turn, it can be seen that the failure to *trans*-methylate at C-24 in *erg6* does not prevent the subsequent trans-

formations. Zymosterol is the most abundant sterol in this strain but the presence of $\Delta^{5,7,24}_{C}$ and $\Delta^{5,7,22,24}_{C}$, which are analogous to ergosterol and 24(28) dehydroergosterol in wild type, implies that cholestanes are reasonable substrates for the Δ^8 to Δ^7 isomerase, 5(6) desaturase, and the 22(23) desaturase.

The major sterol in *erg2* is fecosterol ($\Delta^{8,24(28)}_{E}$) and its immediate precursor, zymosterol, is not detectable. This is surprising since zymosterol is a major accumulation in wild type. It is also noticeable that *erg2* has no sterols with side chain dienes yet accumulates both $\Delta^{8,22}_{E}$ and $\Delta^{5,8,22}_{E}$ suggesting that the 24(28) desaturase is effective with Δ^8 sterols as substrates. The absence of zymosterol in this strain could imply that the C_{24} transmethylase is subject to control by either Δ^{7}_{E} or $\Delta^{5,7}_{E}$ sterols.

Erg3 resembles *erg2* in that it also does not contain detectable levels of zymosterol, suggesting that the C_{24} transmethylase may be controlled by $\Delta^{5,7}_{E}$ sterols. In this mutant, Δ^{7}_{E} sterols are relatively more abundant than Δ^{8}_{E} sterols; however, the complexity of the mixture made it difficult to quantify them in any more meaningful manner. *Erg3* does accumulate sterols with side chain dienes, $\Delta^{8,22,24(28)}_{E}$ and $\Delta^{7,22,24(28)}_{E}$. In strains carrying the nonleaky *erg3* allele, which was used in the present study, the accumulation of $\Delta^{22,24(28)}_{E}$ sterols is independent of cultural conditions and respiratory competence; whereas, with the leaky mutants used in earlier work (11,12), such sterols are not detectable in log phase cultures or petite derivatives. At present we can offer no explanation for these observations.

On a priori grounds, *erg5* would be expected to accumulate $\Delta^{8,24}_{C}$, $\Delta^{7,24(28)}_{E}$, $\Delta^{5,7,24(28)}_{E}$, and $\Delta^{5,7}_{E}$. Although $\Delta^{5,7,24(28)}_{E}$ was not available as a standard, its retention characteristics can be calculated from the data in Table II; viz.; SE-30, 3.71; OV-17, 1.48; OV-225, 1.63. *Erg5* does give a clear peak at 1.63 on OV.225 (see Table V), but the major peaks on SE-30 (3.79) and OV-17 (1.46 would both encompass $\Delta^{5,7}_{E}$ and $\Delta^{5,7,24(28)}_{E}$. Zymosterol is detectable by by GLC in this mutant.

In the three double mutants involving *erg6*, the major sterol accumulated had the retention characteristics of zymosterol and all other peaks were small by comparison. In both *erg2/3* and *erg2/5*, the major accumulation appeared to be $\Delta^{8,24(28)}_{E}$ and zymosterol was not detected. The sterols in *erg3/5* were in accord with expectation. The active enzymes in this genotype are the C-24 transmethylase, the Δ^7 to Δ^8 isomerase, and the 24(28) desaturase, allowing the sequence

$$\Delta^{8,24}_{C} \rightarrow \Delta^{8,24(28)}_{E} \rightarrow \Delta^{7,24(28)}_{E}$$
$$\downarrow \qquad\qquad \downarrow$$
$$\Delta^{8}_{E} \qquad\qquad \Delta^{7}_{E}$$

and these were the sterols detected. Zymosterol was present in low quantities relative to fecosterol and episterol.

This analysis clearly shows that the terminal steps of sterol biosynthesis are not necessarily sequential since the enzymes concerned seem to have a broad structural specificity. However, it is also clear that the reactions are not completely independent and that the metabolic blocks in the mutants place some restrictions on the operation of unaffected enzymes. Moore and Gaylor (26) have shown that the activity of the C-24 transmethylase is controlled by $\Delta^{5,7}$ sterols. Our results indicate that, in the absence of such molecules, the conversion of zymosterol to fecosterol is uncontrolled insofar as little or no zymosterol accumulates even though the total sterol content may be 2-4 times that of wild type. It is likely that further studies with the *erg* mutants will add considerably to our knowledge of the control of sterol biosynthesis in yeast.

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Cysteine Adds to Lipid Hydroperoxide

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ABSTRACT

Cysteine reacts with linoleic acid hydroperoxide to yield several products, some of which were identified as fatty acid-cysteine adducts. The addition was catalyzed by ferric chloride (10^{-5} M) by initiating free radical reactions. When isomerically pure 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid and cysteine were reacted in 80% ethanol under N_2, the major adducts were 9-S-cysteine-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid (I) and 9-S-cysteine-10,13-dihydroxy-*trans*-11-octadecenoic acid (II). When the reaction included both isomers of the hydroperoxide (13- and 9-hydroperoxide) and air, an adduct of 9-oxononanoic acid and cysteine also was isolated. Additional experiments gave information about possible mechanisms of I and II formation.

INTRODUCTION

Often lipid hydroperoxides have a deleterious effect on proteins through modification of their structure by oxidation and addition reactions. Investigations have shown that sulfhydryl enzymes are particularly susceptible to inactivation by peroxidizing lipids (1,2). On the other hand, lipid hydroperoxides have a variable effect on the activity of nonsulfhydryl enzymes (2-4). Analyses of amino acid residues from enzymes and other proteins treated with lipid hydroperoxides demonstrated that cysteine-cystine is one of the most labile residues (4,5). Schaich and Karel (6) studied the reaction of both cystine and cysteine with peroxidizing methyl linoleate by electron spin resonance spectroscopy and discovered that cysteine yielded radical signals much more readily than cystine. Their data suggested that the thiol of cysteine was converted to a sulfur-oxygen complex rather than a thiyl radical.

A number of researchers have used model systems to assess cysteine damage. Some of the cysteine reactions characterized are addition to malonaldehyde (7), oxidation to cystine and higher oxidation states (5,8), and addition to lipid hydroperoxide (9). In the latter study, a specific isomer of linoleic acid hydroperoxide, 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid, was reacted with N-acetylcysteine yielding adducts which were identified as 9-S-(N-acetylcysteine)-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid and 9-S-(N-acetylcysteine)-10,13-dihydroxy-*trans*-11-octadecenoic acid. In this communication, we report equivalent adducts of cysteine with fatty acid. An adduct of cysteine and 9-oxononanoic acid also was identified, which required the participation of the free amino group. A previous study of the linoleic acid hydroperoxide-cysteine reaction (10) had focused on identification of the oxygenated fatty acids produced rather than the cysteine-fatty acid adducts reported here.

METHODS

Reaction Conditions and Isolation of Products

13-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was isolated according to Gardner (11) and reacted with cysteine in 80% ethanol as described previously (10). The reaction catalyst was 10^{-5} M $FeCl_3$. A nitrogen atmosphere minimized competing reactions with oxygen and assured higher yields of adducts (9). The products were dissolved in 4 ml $CHCl_3$ with sufficient CH_3OH to effect solution, slurried with 2 g silicic acid, and applied to a column (ID 2.5 cm) packed with 50 g Mallinckrodt silicic acid (100 mesh, analytical reagent) in $CHCl_3$. The column was eluted stepwise with 100 ml $CHCl_3$, 150 ml 2% CH_3OH, 250 ml 10% CH_3OH, 350 ml 20% CH_3OH, 350 ml 30% CH_3OH, and 200 ml 50% CH_3OH in $CHCl_3$. An adduct identified as 9-S-cysteine-13-hydroxy-10-ethoxy-*trans*-11-octadecenoic acid (I) eluted between volumes 640 and 860 ml, and 9-S-cysteine-10,13-dihydroxy-*trans*-11-octadecenoic acid (II) eluted between 1000 and 1130 ml. A third compound, subsequently identified as an adduct of cysteine and 9-oxononanoic acid, eluted between volumes 410 and 510 ml. This product was treated with CH_2N_2, and the resultant dimethyl ester was designated as III. The structures of I, II, and III are shown in Figure 1.

Eluants from the column were detected by thin layer chromatography (TLC) analyses using 250 μ Silica Gel G plates with $CHCl_3$-CH_3OH-H_2O-CH_3COOH 65:25:4:1 as developing solvent in a filter-paper lined tank [R_{f_I} = 0.38, $R_{f_{II}}$ = 0.24, $R_{f_{III}}$ (free acid) = 0.41].

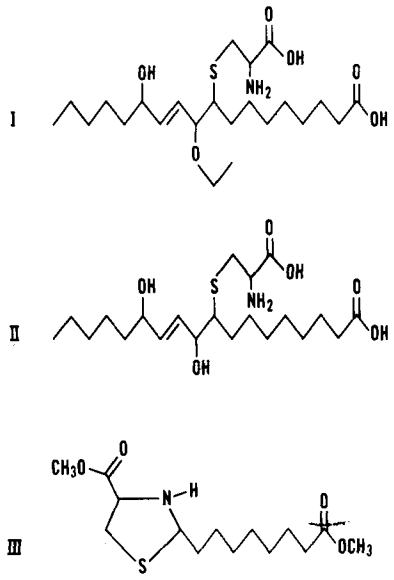


FIG. 1. Numerical key and structures of products from the reaction of cysteine with linoleic acid hydroperoxide.

In a second reaction, conditions and substrate were different from those described above. The reaction differed in that the hydroperoxide was a mixture of isomers [9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic acid (21%) and 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (79%)], and that the reaction proceeded in the air rather than under nitrogen.

Synthesis and Isolation of III

In order to identify III, this compound was synthesized by a known route. We reacted methyl 9-oxononanoate with cysteine, and subsequently treated this product with CH_2N_2 to obtain III. Methyl 9-oxononanoate was obtained by oxidation of methyl 9,10-dihydroxystearate (Analabs, North Haven, CT 06473) with periodic acid. Methyl dihydroxystearate (1 g) was dissolved in 52 ml tetrahydrofuran, and to this solution was added 35 ml 1 N H_2SO_4 containing 0.83 g periodic acid. After 10 min, the reaction was diluted with an equal volume of H_2O and extracted with ether. The ether layer, washed twice with H_2O, yielded a mixture of methyl 9-oxononanoate and nonanal. Initially, we isolated the 9-oxononanoate by preparative TLC, but soon abandoned this time-consuming procedure. Instead, we used both aldehydes in the reaction with cysteine, and relied on the final chromatographic separation to obtain pure III, as well as the analogous adduct of cysteine and nonanal.

The mixture of aldehydes from above was added to 900 ml 80% methanol containing cysteine (25.6 mM), and this mixture was allowed to stand 24 hr at 25 C. The products were dissolved in ether-$CHCl_3$-CH_3OH (45:45:30) and esterified with CH_2N_2; care was taken to prevent an excess of CH_2N_2 from accumulating. Excess CH_2N_2 causes an unknown compound to form, presumably a methyl derivative of the imino group.

The esterified products containing III were separated by chromatography employing a column (ID 2.5 cm) packed with 50 g Mallinckrodt SiliCAR CC7 in $CHCl_3$. Stepwise elution was with 300 ml $CHCl_3$ and 300 ml 1% CH_3OH in $CHCl_3$. Pure III eluted between 250 and 350 ml; the analogous adduct of cysteine and nonanal eluted pure between 170 and 180 ml; and most other fractions contained mixtures.

Spectral Methods

Mass spectroscopy (MS) was done in tandem with gas liquid chromatography (GC-MS) as described previously (10). I and II were converted to the dimethyl ester and trimethylsilyloxy ethers (10). Subsequently, the derivatized samples were analyzed by GC-MS using a 4 ft x 1/8 in. 3% Dexsil 300 on Gas Chrom Q column programmed from 175-250 C at 4 C/min, and then held isothermally at 250 C. III was analyzed by GC-MS using a 4 ft x 4 mm 3% OV-1 on Gas Chrom Q column programmed from 200-260 C at 4 C/min.

Nuclear magnetic resonance (NMR) and infrared (IR) spectra were obtained as before (10).

RESULTS AND DISCUSSION

Product III

When pure 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was reacted with cysteine under N_2, no discernible III eluted from the silicic acid column. After methyl esterification, the appropriate column fractions were subjected to GC-MS. Evidence for III was ambiguous and weak; thus, it was concluded that if III was present, it was only a trace quantity.

Compound III was recovered in a relatively high yield when a mixture of 21% 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoic and 79% 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid was reacted in the air. It is not known if air or the mixture of isomers is important. Presently, we are studying a number of variables in the reaction of linoleic acid hydroperoxide with cysteine, and this study

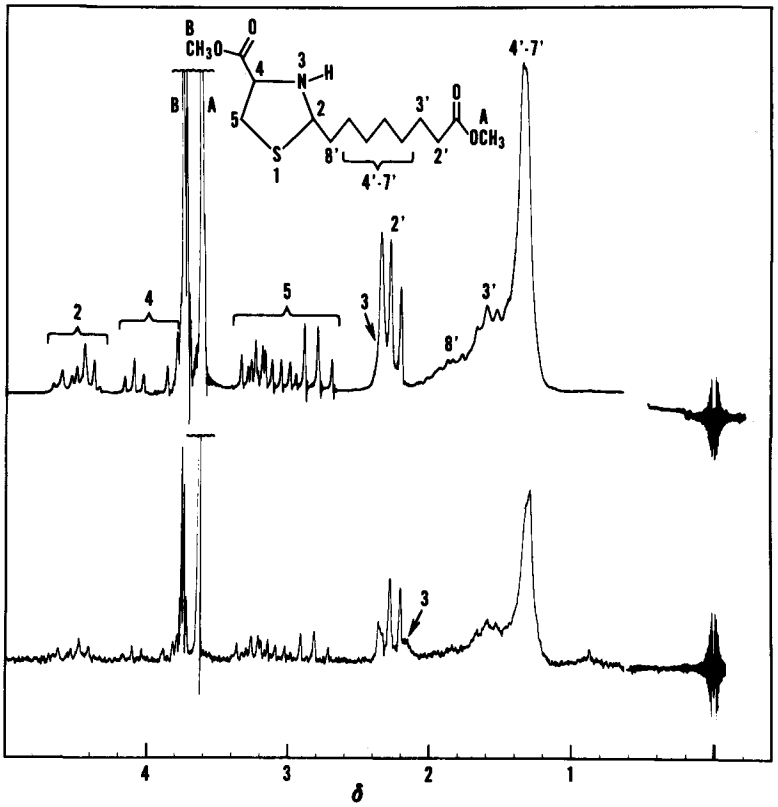


FIG. 2. Nuclear magnetic resonance spectrum of III. Top: synthesized. Bottom: isolated from a cysteine-lipid hydroperoxide reaction.

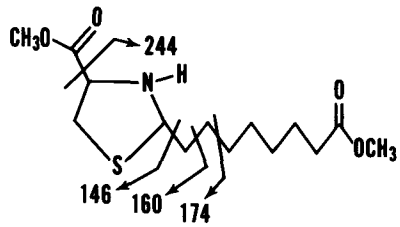
may elucidate further which factors are necessary to the production of III. The appropriate silicic acid column fractions (contained 10% of the total lipid products) were esterified with CH_2N_2, and III was isolated from TLC after double development in $CHCl_3$. Sulfur content was analyzed as follows: calculated for III ($C_{14}H_{25}O_4NS$), S 10.57; found S 10.37.

Figure 2 shows the NMR spectrum of III compared with the spectrum of a III synthesized with known reagents. The NMR spectra were consistent with a 2-substituted thiazolidine-4-carboxylate derivative of 9-oxononanoate. Since the ring is diastereoisomeric, the absorptions of the ring protons are complex. If III was isomerically pure, the absorption of ester methoxy protons of the 4-carboxylate would have been a clean singlet; however, it was not indicating an isomeric mixture. Differences in the configuration of ring substituents readily can be detected by NMR (12). Decoupling experiments with synthetic III revealed that the compound was indeed an isomeric mixture. The proton at C-4 absorbed as an apparent triplet centered at 4.10 δ and another apparent triplet at about 3.79 δ. Irradiation of the C-4 absorption at 3.79 δ decoupled it from the upfield portion of the complex multiplet due to the C-5 protons, revealing a doublet of doublets centered at 2.84 δ, but the downfield C-5 absorptions were little affected. Irradiation of the C-4 proton at 4.10 δ, presumably due to the other isomer, simplified the complex C-5 multiplet centered at about 3.18 δ. The two apparent triplet absorptions at 4.45 and 4.61 δ were assigned to the C-2 proton, since they could be decoupled by irradiation of the C-8' protons. The NH absorption was variable between 2.17 and 2.38 δ and was superimposed on the C-2' triplet. D_2O did not exchange with the NH proton, but did move the absorption slightly downfield; no other effect was noted with D_2O. We further elucidated the NH absorption by examining the corresponding adduct of cysteine and nonanal. An NMR spectrum of the nonanal derivative showed a broad singlet NH-absorption at 2.24 δ unobscured by the C-2' triplet. The spectral features due to the protons about the thiazolidine ring were identical for both III and the adduct of nonanal and cysteine, methyl ester.

MS spectra of the synthetic and isolated III

TABLE I

Intense Ions Observed in Mass Spectra of III



Ion (m/e)	Possible origin	Relative intensity (%)	
		Isolated	Synthetic
41		10.3	10.7
43		9.5	9.8
55		20.9	19.9
59		15.6	17.4
67		6.7	6.3
75		5.8	6.3
86		23.2	27.3
102		7.7	9.5
114	(146-32)	8.5	9.7
137		4.9	5.0
146		100.0	100.0
160		6.9	2.4
174		6.9	7.7
184	$+N=CH(CH_2)_7COOCH_3$	6.2	7.1
212	(M-59-32)	7.0	6.1
244	(M-59)	18.3	20.0
256	(M-32-15)	13.9	15.1
272	(M-31)	7.6	8.3
304	(M+1)	2.1	4.4

were virtually identical (Table I). The fragment ions confirmed the proposed structure. The IR spectral assignments of III are: 3300 cm^{-1} (weak), secondary amine; 1740 cm^{-1} (strong), ester carbonyl; 1020 cm^{-1} (medium), CH_2-S; 790 cm^{-1} (medium), NH rock; and 2920, 2850 cm^{-1} (strong), CH.

Cysteine will form thiazolidinecarboxylic acid derivatives with aldehydes under the conditions used in this study (13), and probably this was the origin of III. Apparently, 9-oxononanoic acid was produced in this reaction of cysteine and linoleic acid hydroperoxide, and subsequently the cysteine reacted with the 9-oxononanoic acid. A number of other aldehydes are derived from the decomposition of linoleic acid hydroperoxide (14), and thus other aldehyde-cysteine adducts could be present; however, this possibility was not investigated in depth. A number of compounds separated by GC-MS had significant 146 m/e fragment ions which is diagnostic of the thiazolidine-4-carboxylate ring. The production of III and possibly other similar compounds emphasized the potential for hydroperoxide-derived aldehydes to alkylate cysteine; however, the possibility of formation of the thiazolidine ring in proteins would be limited only to proteins having an N-terminal cysteine residue.

Products I and II

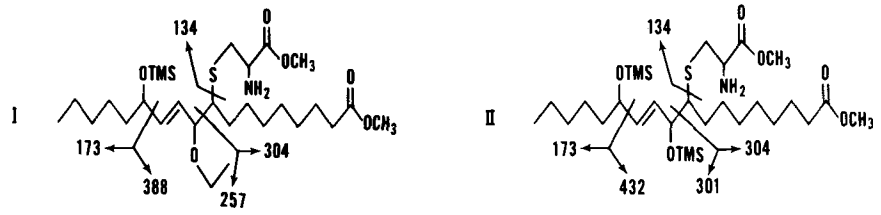
I and II were isolated from the reaction of 13-hydroperoxyoctadecadienoic acid with cysteine. The catalyst, 10^{-5} M $FeCl_3$, was necessary to initiate free radical formation, and the reaction was kept under nitrogen to enhance yields of I and II. Under these conditions, I and II comprised 29% and 7.4% of the lipid products, respectively.

The structures of I and II were ascertained by GC-MS of their derivatives, dimethyl esters-trimethylsilyloxy ethers. MS (Table II) showed that the substituent groups of I and II added identically to that observed in a previous study (9) when N-acetylcysteine was used in place of cysteine. Possibilities exist for erythro-threo isomerism at carbons-9 and -10; occasionally, two barely separable spots were detected on TLC for I and II, which lends support for this possibility.

A proposed pathway to formation of I and II was suggested previously (9). This mechanism proposed that hydroxyoctadecadienoic acid was intermediate to formation of I and II. In

TABLE II

Intense Ions Observed in Mass Spectra of I and II



Ion (m/e)	Possible origin	Relative intensity (%)	Ion (m/e)	Possible origin	Relative intensity (%)
73	TMS	100	73	TMS	100
75	TMS	27.6	75	TMS	48.0
173		36.2	173		15.6
211	257-46	13.4	211	301-90	28.4
254	388-134	7.1	301		9.4
257		29.5	304		14.4
304		62.1	381	M - (134 + 90)	1.0
355	M - 206	4.2	399	M - 206	2.3
381	M - (134 + 46)	4.6	470	M - (134 + 1)	0.2
388		0.3			
426	M - (134 + 1)	2.0			
515	M - 46	0.6			

one experiment, we substituted 13-hydroxy-*cis*-9,*trans*-11-octadecadienoic acid and a 2.3 molar excess of H_2O_2 (relative to hydroxyoctadecadienoic acid) for 13-hydroperoxyoctadecadienoic acid in the reaction. All other conditions remained the same as before (under N_2). If hydroxyoctadecadienoic acid is intermediate, H_2O_2 should supply the necessary peroxide to initiate addition by cysteine thiyl radical. Adducts were observed, but all products were not the same as with hydroperoxide as a reactant. The major product was more polar than I and II. Although evidence for I was absent, a compound was found with chromatographic properties (column and GC) identical to II. This compound yielded an MS spectrum that was virtually identical to the spectrum obtained from authentic II. The only difference was the appearance of two additional fragment ions (m/e 259 and 313), both of which were less than 10% relative intensity. These two ions have been noted before when MS were obtained of II isolated from a reaction involving both 9- and 13-hydroperoxyoctadecadienoic acid isomers and cysteine; however, MS of this mixture gave other significant ions not observed in the spectrum in question. The ion, 259 m/e, is typical of fatty acids having a 9-trimethylsilyloxy group. Thus, one could surmise that the addition was somewhat less specific, and that a few hydroxy groups added to carbon-9. Since H_2O_2 and $FeCl_3$ (Fenton's reagent) is known to produce hydroxyl radicals, an anti-Markownikoff addition of hydroxy could occur at carbon-9. Considering that the major product was more polar, it seems plausible that H_2O_2 gives rise to more extensive radical attack and less specific products. Despite the ambiguous result, II was synthesized from hydroxyoctadecadienoic acid, and it seems plausible that it could serve as an intermediate.

In another experiment, the participation of solvent was made obvious. When 80% methanol was used as a solvent instead of 80% ethanol, a 10-methoxy group replaced the 10-ethoxy of I. The MS of the methoxy derivative corresponded to that of I, except the appropriate fragment ions appeared 14 amu lower.

Since I and II were significant products of the reaction, it is likely that this type of adduct formation could be very important in both blocking protein sulfhydryl groups and the formation of lipid-protein complexes. Roubal and Tappel (15) observed lipid-protein complexes after reacting protein with peroxidizing lipids.

Other Products

The products from the hydroperoxide-cysteine reaction contained lipids other than I and II that gave a positive reaction to ninhydrin, but they were relatively small quantities.

In the water solubles, cystine was identified by amino acid analysis. Because cysteine inter-

feres in the method used (16), prior treatment of the water solubles with 4-vinylpyridine was necessary (17).

Inevitably, each product mixture was colored brown. When attempts were made to isolate the "brown" compounds, it was discovered that they were a number of different compounds amounting to a very minor quantity. Since the product mixture from the use of N-acetylcysteine instead of cysteine is not brown, the amino group is implicated in the browning reaction. Evidently, the amino group is reactive with the secondary products of hydroperoxide degradation.

ACKNOWLEDGMENTS

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Metabolism of Arachidonate and Stearate Injected Simultaneously into the Mouse Brain

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ABSTRACT

The metabolism of a polyunsaturated and a saturated fatty acid in brain membrane phosphoglycerides was examined by injecting simultaneously a mixture of ^{14}C-arachidonate and ^{3}H-stearate into the mouse brain and isolating the microsomal and synaptosomal fractions at 1-40 min after injections. Both types of labeled fatty acids were utilized more readily in the microsomal than the synaptosomal fractions in brain. However, labeled arachidonate was incorporated more rapidly into membrane phosphoglycerides than was stearate. In both subcellular fractions, the relative specific radioactivity (^{3}H and ^{14}C) of diacyl-glycerophosphorylinositol (diacyl-GPI) was higher than other types of phosphoglycerides such as diacyl-glycerophosphorylcholine (diacyl-GPC) and diacyl-glycerophosphorylethanolamine (diacyl-GPE). Furthermore, the apparent rates of incorporation of radioactivity into diacyl-GPI was more rapid for the ^{14}C-arachidonate than for the ^{3}H-stearate. Results of the experiment have demonstrated obvious differences in metabolism between stearate and arachidonate in brain. The more rapid transfer of arachidonate to diacyl-GPI is probably due to the presence of an acyl transferase system specially active for the transfer of arachidonyl groups to diacyl-GPI.

INTRODUCTION

The free fatty acids (FFA) in brain are known to be metabolically active and are in dynamic equilibrium with the acyl groups of membrane glycerolipids. In vivo experiments have demonstrated that labeled fatty acids injected intracerebrally into the mouse brain were rapidly incorporated into the glycerolipids (1-3). The incorporation of fatty acids into membrane phospholipids may proceed either through the de novo pathway via phosphatidic acids and diacylglycerols as intermediates (4) or a direct acylation mechanism involving lysophosphoglycerides as acceptor molecules (5-7). However, the extent and participation of the different pathways in brain have not been investigated extensively. Recent in vitro studies with brain microsomes (6) and synaptosomes (7) have indicated that the direct acyl transfer mechanism may play an important role in directing acyl group metabolism of brain membrane phosphoglycerides for the enzyme exhibits specificity towards the acyl groups as well as the chemical nature of the acceptor molecules. Since different types of fatty acids are present in the in vivo system, a comparison of the metabolic behavior of the saturated with the polyunsaturated fatty acids in brain may yield additional information concerning the extent of the different acylation mechanisms. In the present study, we have injected intracerebrally into the mouse brain a mixture containing ^{3}H-stearate and ^{14}C-arachidonate. Their incorporation into individual phosphoglycerides in subcellular brain membranes was examined as a function of time.

MATERIALS AND METHODS

Animals and Mode of Administration

Approximately 20 adult C57BL/10J mice were used for the experiment. For preparation of labeled precursors, a hexane solution of 100 μCi (9,10-^{3}H)-stearic acid (0.5 Ci/nmole, radiochemical purity >99%, Applied Science Laboratories, State College, PA) was mixed with a solution of 50 μCi (1-^{14}C)-arachidonic acid (55.5 mCi/nmmole, radiochemical purity >94%; Applied Science Laboratories). The amount of labeled fatty acids injected into the mouse brain has been tested previously not to exceed the brain FFA pool. After evaporation of the organic solvent, the mixture of labeled fatty acids was complexed with bovine serum albumin (10 mg/ml) in 0.32 M sucrose solution containing 50 mM Tris (pH 7.4). Each mouse was injected intracerebrally with 20 μl of the mixture (2), and they were sacrificed in groups of three at 1, 3, 6, 10, 20, and 40 min after injections. Normally, a time period of 1 min was allowed for decapitation, dissection, and dispersion of the brain tissue and this time was not included in the time interval described above. Immediately following dissection, each mouse brain was dispersed in 20 vol of ice cold 0.32 M sucrose solution containing 1 mM

TABLE I

An Estimation of the Endogenous Levels of Arachidonate and Stearate in Individual Phosphoglycerides of Mouse Brain Subcellular Fractions[a]

Phosphoglycerides	Synaptosome-rich fraction						Microsomal fraction					
	% wt		Phosphoglycerides		20:4	18:0	Phosphoglycerides		20:4	18:0		
	20:4	18:0	%, mole	nmole/brain	nmole/brain		%, mole	nmole/brain	nmole/brain			
diacyl-GPI	31.5	35.8	3.4	160	50	57	5.0	46	15	17		
diacyl-GPS	2.3	50.1	14.3	670	15	335	11.2	103	2	52		
diacyl-GPC	6.0	14.3	38.6	1810	109	259	38.9	358	22	51		
alkenylacyl-GPE	18.6	3.8	17.6	820	153	31	18.3	168	31	6		
diacyl-GPE	14.2	35.9	18.6	870	124	312	19.8	182	26	66		

[a]The percentage wt distribution of arachidonate and stearate in individual phosphoglycerides was obtained by gas liquid chromatography (GLC) analysis of their acyl groups after alkaline-methanolysis. Individual phosphoglycerides were separated by two-dimentional thin layer chromatography (TLC) as described in text. The same percentage acyl group distribution was used for both subcellular fractions because results did not indicate obvious differences in distribution. The amount of phosphoglycerides in each fraction was determined by assaying the lipid-phosphorus content after two-dimensional TLC separations. The amount of phospholipids in the subcellular fractions was based on the sum of all the phospholipids recovered from the thin-layer plate. Values are means of four determinations from four brain samples.

[b]Abbreviations: GPI = glycerophosphorylinositol, GPS = glycerophosphorylserine, GPC = glycerophosphorylcholine, GPE = glycerophosphorylethanolamine.

EDTA, 1 mM $MgCl_2$, and 50 mM Tris buffer, pH 7.4. A portion of each brain dispersion was taken for measurement of the radioactivity.

Subcellular Fractionation, Lipid Extraction and Separation

The procedure for isolating subcellular fractions from the brain dispersion has been described (9). In the present experiment, only microsomes and synaptosome-rich fractions were isolated since results from previous experiments (3) have indicated that a negligible amount of the labeled fatty acids was incorporated into the myelin membranes during this short time interval. The purity of the subcellular fractions and characterization of protein and lipid composition of these fractions have been described (3). The procedures for lipid extraction and separation of individual lipid components by two-dimensional thin layer chromatography have been described (2,10).

The radioactivity of lipid samples containing both (^3H) and (^{14}C) was measured with a Packard Liquid Scintillation Spectrometer (Model 3385) with an automatic external standard device. Tritium was counted by a channel with window preset for measuring ^3H in the presence of ^{14}C material. Similarly, radioactivity of ^{14}C was counted with a channel preset for measuring ^{14}C in the presence of ^3H. Less than 2% of the tritium was counted in the ^{14}C-channel. However, a small but constant proportion (ca. 10%) of ^{14}C was detected in the tritium channel. With this setting, the ^3H:^{14}C ratio (cpm) of the fatty acid mixture prior to injection was 3.5.

RESULTS

An estimation of the endogenous levels of stearate and arachidonate in individual phosphoglycerides of the brain subcellular fractions is shown in Table I. Based on lipid phosphorus determinations, phospholipids recovered in the synaptosome-rich fraction were normally 5 times higher than those in the microsomal fraction. The acyl group composition of individual phosphoglycerides in the microsomal and synaptosomal fractions was similar. There is normally more stearate esterified to the brain glycerolipids than arachidonate. However, the diacyl-glycerophosphorylinositol (diacyl-GPI) in both subcellular fractions have equal proportions of arachidonate and stearate whereas the acyl groups of diacyl-glycerophosphorylcholine (diacyl-GPC) have more stearate than arachidonate.

After intracerebral injection of the fatty acid mixture, radioactivity from both types of

TABLE II

The Percent Radioactivity Remaining as Labeled Free Fatty Acid in Microsomes and Synaptosomal Fractions of Mouse Brain after Simultaneous Intracerebral Injection of ^{3}H-stearate and ^{14}C-arachidonate[a]

Time (min)	^{3}H-stearate		^{14}C-arachidonate	
	Microsomes	Synaptosomes	Microsomes	Synaptosomes
1	90.5	92.6	91.2	88.1
3	80.2	86.3	76.3	78.1
6	70.6	85.1	61.4	70.1
10	63.9	81.1	51.1	66.8
20	43.9	70.3	31.1	43.8
40	29.6	60.4	21.5	28.5

[a]Results are obtained by measuring the radioactivity of the free fatty acid spot and comparing with total radioactivity from other lipid spots on the thin layer chromatography (TLC) plates. Values are means from three samples for each time interval.

labeled precursors was incorporated into the diacyl-GPC and diacyl-GPI with smaller proportions in diacyl-glycerophosphorylethanolamine (diacyl-GPE) and triacylglycerols. In spite of the high proportions of arachidonoyl group in alkenylacyl-GPE and stearoyl group in diacylglycerophosphorylserine (diacyl-GPS), these phosphoglycerides were not labeled appreciably during the initial time periods and were therefore not included in the present study.

Results in Table II show the proportion of radioactivity of labeled fatty acids remaining unesterified in the brain after injections. The rate of disappearance of the radioactivity of free fatty acids gave indication as to the extent of utilization of labeled precursors by the glycerolipids. Results showed that fatty acid utilization in the microsomal fraction was more rapid than that in the synaptosome-rich fraction. By comparing the decrease in radioactivity, it is also apparent that labeled arachidonate was turning over more rapidly than stearate in the respective subcellular fractions. Within the time of the experiment, most of the labeled fatty acids were remaining in the original form as shown by result of analysis by $AgNO_3$-argentation TLC. Less than 2% of conversion to other acyl groups was observed for the labeled stearate (Wise and Sun, unpublished data).

Among the various phosphoglycerides examined, specific radioactivity of diacyl-GPI was highest with regard to either ^{3}H or ^{14}C labeling and in both subcellular fractions (Figs. 1 and 2). In the microsomal fraction (Fig. 1), the incorporation of ^{3}H-stearate into diacyl-GPI, diacyl-GPC, and diacyl-GPE increased with time (upper graph). The incorporation of ^{14}C-arachidonate into diacyl-GPI, however, showed a rapid increase in the initial 10 min and started to decline afterwards (lower graph). In the synaptosomal fraction (Fig. 2), a significant

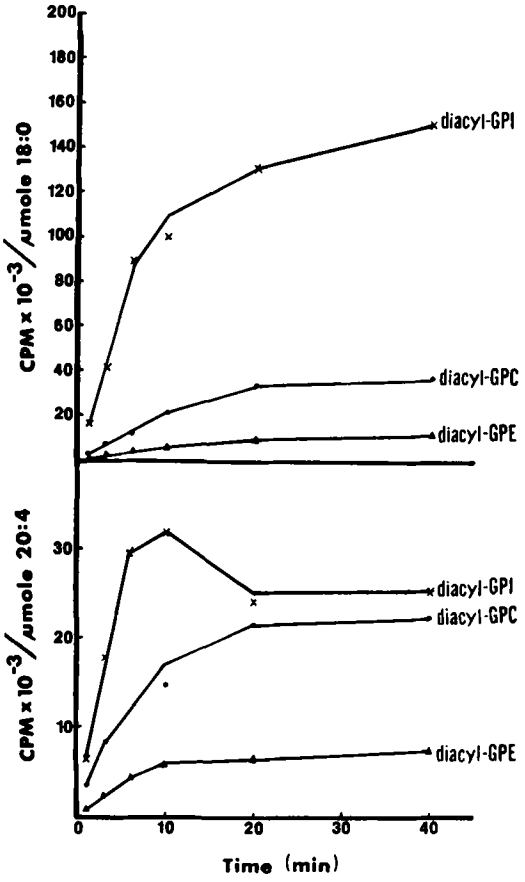

FIG. 1. Specific radioactivity of phosphoglycerides in mouse brain microsomal fractions after simultaneous intracerebral injections of (1-^{14}C)-arachidonic and (9,10-^{3}H)-stearic acids.

portion of the radioactivity (both ^{3}H and ^{14}C) was present in diacyl-GPI at 1 min after injection indicating a more rapid uptake of labeled precursors for diacyl-GPI than for diacyl-GPC

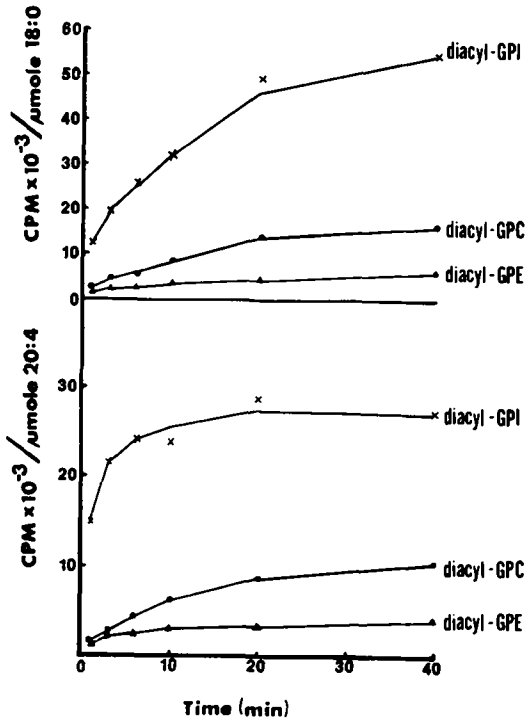

FIG. 2. Specific radioactivity of phosphoglycerides in mouse brain synaptosome-rich fractions after simultaneous intracerebral injections of (1-^{14}C)-arachidonic and (9,10-^{3}H)-stearic acids.

TABLE III

The ^{3}H:^{14}C Ratios of Individual Lipids in Mouse Brain Subcellular Fractions after Simultaneous Intracerebral Injections of (^{3}H)-stearate and (^{14}C)-arachidonate

Time (min)	diacyl-GPI	diacyl-GPC	diacyl-GPE	FFA[a]
		Microsomes		
1	1.2	2.0	2.7	2.5
3	1.1	2.2	2.5	3.3
6	1.4	4.3	2.9	3.8
10	1.7	3.6	3.1	3.6
20	2.6	3.6	3.7	5.5
40	2.8	3.8	4.2	5.7
		Synaptosomes		
1	0.9	4.4	2.2	4.0
3	1.1	3.3	2.5	4.5
6	1.2	2.9	2.5	5.2
10	1.5	3.3	2.7	5.5
20	2.0	3.7	3.4	8.2
40	2.3	3.7	3.8	11.6

[a]Denotes ratios of radioactivity of labeled precursor remaining in the subcellular fractions after intracerebral injections. Abbreviations: GPI = glycerophosphorylinositol, GPC = glycerophosphorylcholine, GPE = glycerophosphorylethanolamine, FFA = free fatty acid.

TABLE IV

Ratios of Radioactivity Comparing diacyl-GPI and diacyl-GPE with diacyl-GPC at Various Times after Intracerebral Injections of (9,10-^{3}H)-stearate and (1-^{14}C)-arachidonate[a]

	diacyl-GPI / diacyl-GPC		diacyl-GPE / diacyl-GPC	
Time (min)	^{14}C-A	^{3}H-S	^{14}C-A	^{3}H-S
		Microsomes		
1	1.3	0.8	0.3	0.4
3	1.6	0.7	0.4	0.4
6	2.4	0.9	0.8	0.5
10	1.2	0.6	0.5	0.4
20	0.8	0.6	0.4	0.4
40	0.8	0.6	0.4	0.4
		Synaptosomes		
1	5.0	1.0	1.4	0.7
3	3.3	1.1	0.8	0.6
6	2.4	1.0	0.6	0.5
10	1.4	0.8	0.6	0.5
20	1.5	0.8	0.4	0.4
40	1.3	0.8	0.5	0.5

[a]Diacyl-GPI = diacyl-glycerophosphorylinositol, diacyl-GPE = diacyl-glycerophosphorylethanolamine, diacyl-GPC = diacyl-glycerophosphorylcholine, A = arachidonic acid, and S = stearic acid.

and diacyl-GPE. The incorporation of ^{14}C-arachidonate into diacyl-GPI seemed to be completed within 20 min (lower graph) since no further increase in radioactivity was observed between 20 and 40 min.

The ^{3}H:^{14}C ratios of individual phosphoglycerides and FFA expressed as a function of time are shown in Table III. In general, ^{3}H:^{14}C ratios seemed to increase with time, beginning with values lower than 3.5 and ending with higher values. Although the ^{3}H:^{14}C ratios for diacyl-GPI increased with time, they were consistently lower than those for diacyl-GPC and diacyl-GPE indicating a difference in the entry of labeled fatty acids between the two types of phosphoglycerides.

The incorporation of labeled arachidonate into diacyl-GPI and diacyl-GPC was examined by comparing the ^{14}C-ratios at various times after incorporation (Table IV). In the microsomal fraction, the values of diacyl-GPI to diacyl-GPC ratios (^{14}C) increased between 1 and 6 min and then decreased afterwards. However, the same type of ratios in the synaptosomal fraction were highest at 1 min and decreased approximately threefold during the 40 min interval. Changes in ^{3}H-ratios between diacyl-GPI and diacyl-GPC were similar in both subcellular fractions, showing a slight decrease in values at 6 min after injection. There was essentially no change in ^{3}H-ratios between diacyl-GPE and diacyl-GPC in the microsomal

fraction and in most time points shown in the synaptosomal fraction. The time changes in ^{14}C-ratios between diacyl-GPE and diacyl-GPC in the subcellular fractions resembled closely those obtained between diacyl-GPI and diacyl-GPC.

DISCUSSION

In the present experiment, differences in the metabolism of stearate and arachidonate in brain can be demonstrated by examining the incorporation of radioactivity (^{3}H and ^{14}C) into the membrane glycerolipids as a function of time. Results indicate that the rate of incorporation of arachidonate into the brain glycerolipids was faster than the stearate. Furthermore, both fatty acid precursors were utilized more readily in the microsomal fraction than the synaptosomal fraction.

By examining the specific radioactivity of phosphoglycerides with time, two types of uptake patterns were observed with regard to arachidonate incorporation into the major phosphoglycerides, i.e., arachidonate incorporation into diacyl-GPI seemed to be more rapid and thus achieving higher specific radioactivity than that for diacyl-GPC and diacyl-GPE. Labeled arachidonate injected into the brain was found to be acylated exclusively to the C-2 position of brain phosphoglycerides (8). Results here are in agreement with experiments in vitro indicating the presence of acyl transferase(s) catalyzing the transfer of unsaturated acyl groups to 1-acyl-phosphoglycerides (6,7). Possibly, the enzyme system here also exhibited a higher specificity for arachidonoyl transfer to diacyl-GPI as compared to diacyl-GPC and diacyl-GPE.

The comparison of the relative rate of uptake of arachidonate and stearate by individual phosphoglycerides gave increasing ^{3}H:^{14}C ratio with time (Table III). The increase in ratio may be explained as due to a comparatively higher proportion of ^{14}C-arachidonate incorporated into the phosphoglycerides at the initial time periods. Since the ^{3}H:^{14}C ratios for the mixture prior to injection was 3.5, the lower ratios for diacyl-GPI further indicates the presence of a more rapid acylation system for arachidonyl transfer to this phosphoglyceride. In the FFA fraction, the increase in ^{3}H:^{14}C ratios may be explained as due to a more rapid utilization of ^{14}C-arachidonate as compared to ^{3}H-stearate in the initial time periods.

Differences in arachidonate and stearate metabolism can be further illustrated by taking the ratios (^{14}C and ^{3}H) of radioactivity between different phosphoglycerides (Table IV). Relatively constant ratios of ^{3}H-stearate incorporation were maintained among the diacyl-GPI, diacyl-GPC, and diacyl-GPE suggesting that the incorporation of stearate into these phosphoglycerides may occur under a similar mechanism. On the other hand, obvious differences were observed with regard to ^{14}C-arachidonate incorporation into diacyl-GPI and to a smaller extent diacyl-GPE as compared to diacyl-GPC. The observation implies that some of the labeled arachidonate incorporated into diacyl-GPE in the initial time periods may be different from that for diacyl-GPC. However, the exact mechanism remains to be investigated.

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Accumulation of Ergosta-8,14-dien-3β-ol by *Saccharomyces cerevisiae* Cultured with an Azasterol Antimycotic Agent

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ABSTRACT

15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol, an antimycotic agent, at a concentration of 75 ng/ml inhibited ergosterol biosynthesis in *Saccharomyces cerevisiae* strain 3701B resulting in the accumulation of an unusual sterol. Experimental data presented indicate that this sterol is ergosta-8,14-dien-3β-ol. The accumulation of the compound is supportive of current models of biosynthetic pathways for sterols in yeast and is consistent with inhibition by the azasterol of the Δ^{14} sterol reductase.

INTRODUCTION

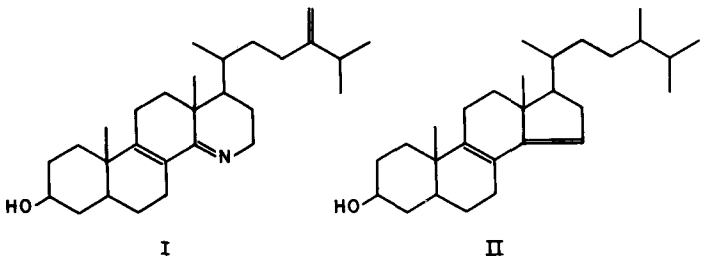
The antimycotic agent 15-aza-24-methylene-D-homocholesta-8,14-dien-3β-ol(I) (1) was found to be a competitive inhibitor of the S-adenosylmethionine:Δ^{24}-sterol methyltransferase both in vivo and in vitro in *Saccharomyces cerevisiae*. Aerobically adapting yeast in the presence of I (200 μg/ml) produced no ergosterol or sterols giving a typical positive Liebermann-Burchard assay (2). The result is unusual, for yeast grown in the presence of 25-azazymosterol, a powerful inhibitor of this enzyme, readily produce C_{27}-$\Delta^{5,7}$ dienes (3). Furthermore, nystatin resistant mutants of *S. cerevisiae* lacking methyltransferase activity have been found to contain C_{27}-$\Delta^{5,7}$ dienes (4,5).

In subsequent experiments, *S. cerevisiae* has been gown in the presence of I at a concentration insufficient for inhibition of the Δ^{24}-methyltransferase and has been found to accumulate a new sterol which typically amounted to more than 90% of the sterols in the nonsaponifiable fraction extracted from treated cultures. In this communication, we report that the new sterol has been identified as ergosta-8,14-dien-3β-ol and briefly comment upon the effects of I on sterol biosynthesis in *S. cerevisiae*.

EXPERIMENTAL PROCEDURES

S. cerevisiae strain 3701B was cultured in 2% tryptone, 1% yeast extract, and 2% dextrose broth at 27 C with shaking. A detailed analysis of the effects of the azasterol on the growth of this organism will be presented elsewhere (6). For labeling studies, L-[methyl-^{14}C] methionine (2 μM, 0.1 μCi/ml) was added to the culture. Azasterol was added as a concentrated solution in 100% ethanol to a final concentration of 75 ng/ml (0.18 μM). Sterols were recovered from the yeast by saponification (17% KOH and 0.14% pyrogallol in methanol-water, 5:2) and extraction with n-hexane. The separation, purification, and acetylation of the sterols were performed as previously described (7). Sterol acetates were also purified by 10% $AgNO_3$-silica gel thin layer chromatography (cyclohexane-benzene, 65:35). Free sterols were recovered by saponification (6% KOH in methanol).

Ultraviolet absorption (UV) spectroscopy was performed on a Cary model 11 recording spectrophotometer. Gas liquid chromatography (GLC) and coupled gas liquid chromatography-mass spectroscopy (GC-MS) have been de-



I II

FIG. 1. 15-Aza-24-methylene-D-homocholesta-8,14-dien-3β-ol (I) and ergosta-8,14-dien-3β-ol (II).

scribed previously (3,8). Quantitative GLC was performed by internal standard analysis using a Varian CDS 111 chromatography data system. Nuclear magnetic resonance (NMR) spectra were obtained on a Varian XL-100 spectrometer in $CDCl_3$ containing tetramethylsilane as internal standard. In labeling studies, radioactivity in isolated sterols was determined by previously described methods (9).

The azasterol, designated A25822B, used in this study was a generous gift of Eli Lilly & Co. (Indianapolis, IN). This is the most abundant member of this group of antimycotic azasterols (10). L-[methyl-^{14}C] methionine was a product of International Chemical and Nuclear Co. (Irvine, CA). Silica gel HF-254 plates were the product of E.M. Laboratories, Inc. (Elmsford, NY). An authentic sample of synthetic ergosta-8,14-dienol was kindly provided by Prof. G.W. Patterson.

On the basis of these data, the major metabolite accumulating in the inhibited yeast was formulated as ergosta-8,14-dien-3β-ol (II). The structural assignment was confirmed by comparison of our data (MS, NMR, and UV) to that obtained from an authentic sample of II (13). The two sets of data were essentially identical, the small differences being attributable to the minor sterol contaminating our sample.

An attractive explanation for the accumulation of II as the end product of sterol metabolism in the inhibited yeast is that, at low concentration (<0.02 μM), I is a specific and potent inhibitor of the Δ^{14}-reductase system. The inability of the yeast to introduce unsaturation at C-22 but to add a methyl group at C-24 in the side chain is consistent with current models of ergosterol biosynthesis in wild type *S. cerevisiae* in which Δ^{22} unsaturation is introduced after $\Delta^{8} \rightarrow \Delta^{7}$ isomerization (14,15).

The present result is similar to the inhibition of the Δ^{24}-reductase of insects (16) by mono- and diazacholesterols having nitrogen(s) in the side chain and of Δ^{14}-reductase in algae and in rat liver homogenates by AY-9944 (17,18). Azasterol I is of particular value, since it is being used to study the sterol Δ^{14}-reductase and the earlier stages of ergosterol biosynthesis in yeast.

RESULTS AND DISCUSSION

Cultivation of the wild type strain 3701B with sublethal concentrations of the azasterol I (75 ng/ml) resulted in cultures containing little or no ergosterol and accumulating another major sterol. In control experiments without the inhibitor, 90% of the total nonsaponifiable lipids was ergosterol. With the azasterol (75 ng/ml), 97% of the total nonsaponifiable lipids was the unusual sterol (6). This compound migrated with the 4-demethyl free sterol fraction on silica gel plates. Isolation of the compound was effected by chromatography of the acetates of the demethyl band on $AgNO_3$-silica gel. The major band was recovered and saponified. Analysis by GLC of the purified 4-demethyl band as the TMS derivative on an OV-101 capillary column (3) revealed the presence of two compounds in a 12:1 ratio.

GC-MS of the free sterol mixture gave a molecule ion peak at m/e 398 (100%) with strong peaks at 383 (70%), 365 (32%), and 271 (22%), which correspond to the empirical formula $C_{28}H_{46}O$ and to loss of $-CH_3$, $-CH_3+H_2O$, and a saturated C_9H_{19} side chain, respectively. The UV spectrum of the purified sterol band in hexane exhibited a λ_{max} of 250 nm with a shoulder at 245 nm, which is characteristic of a sterol containing a $\Delta^{8,14}$ heteroannular conjugated diene (11). Further support for the major metabolite as being a C_{28}-$\Delta^{8,14}$ sterol was provided by the NMR spectrum. The C-18and C-19 methyl resonances appeared at δ 0.82 and 0.99, respectively, which are in good agreement with the calculated shifts of δ 0.83 and 0.94 (12), and compare favorably to those of ergosta-8,14,22-trien-3β-yl benzoate (δ 0.85 and 1.06, respectively) and ergosta-8,14,24(28)-trien-3β-yl benzoate (δ 0.84 and 1.07, respectively) (4). The benzoate has the effect of shifting C-19 absorption downfield ca. 0.27 ppm with respect to the free sterol -3β-OH. A broad one proton absorption at δ 5.33 for the olefinic hydrogen agrees well with a published δ 5.4 absorption for the olefinic hydrogen of the 8,14 conjugation (4).

When 3701B was cultured with L-[methyl-^{14}C] methionine in the presence of subinhibitory levels of the azasterol, the 4-demethyl fraction was labeled. Since sterols are labeled at C-28 by transmethylation during cultivation under these conditions (9), these results indicated that this fraction was C_{28} sterol.

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cis-Enediyne Chromophore of Isano Oil[1]

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ABSTRACT

The nonpolar fatty acids from isano oil have been reinvestigated to determine the source of the UV absorption long attributed to conjugated *cis*-enediyne groupings. Proton magnetic resonance and mass spectra of certain fractions supported the occurrence of such structures, but discrete compounds with this grouping were not isolated. Part of this ubiquituous UV absorption ascribed to enediyne was found to be due instead to the same conjugated diynones identified previously in the oxygenated acid fraction of isano oil. The structures of methyl octadeca-9,11-diynoate and methyl isanate were correlated with their proton magnetic resonance, (PMR), carbon-13 magnetic resonance, and mass spectra.

INTRODUCTION

The fatty acid composition of isano oil has been the subject of much investigation for several decades, culminating in 1963 with the publication of four reports detailing the structures of several acetylenic acids (1-4). The following year a comprehensive two-part review appeared (5,6). Throughout the voluminous literature on isano oil, there have been persistent reports of a ubiquitous UV absorption attributed to a conjugated *cis*-endiyne chromophore. However, isolation from isano oil of a pure acid (or methyl ester) containing this grouping has not been reported except in two cases. In 1957, Meade mentioned the isolation of a *cis*-enediyne (bolekic acid) in the context of a review article (7). Full details of Meade's work were not subsequently published. In 1954, Seher (8) reported the isolation and characterization of a conjugated enediyne (8-hydroxyoctadec-14-ene-10,12-diynoic acid), but this structure was not verified in subsequent investigations of isano oil (1-4,9). Furthermore, there appears to be some doubt about the spectral characteristics of Seher's product. The elegant work of Gunstone (2,3) and Morris (4) did not result in isolation of pure conjugated enediyne acids. On the contrary, this chromophore seemed to be distributed throughout the fractions they obtained.

We undertook a reinvestigation of isano oil in the hope of isolating pure acids responsible for this UV chromophore, and we have reported previously a detailed examination of the polar (i.e., oxygenated) fraction of methyl esters (9). This oxygenated fraction contained two conjugated diynones which accounted for some of the UV absorption. The present paper records our examination of the nonpolar esters of isano oil, particularly in regard to substances responsible for the supposed enediyne chromophore.

EXPERIMENTAL PROCEDURES

Source of Material

Isano oil was purchased from Pacific Vegetable Oil Corporation, San Francisco, CA, and stored at -18 C until examined.

Hydrolysis

Isano oil (19.2 g) was refluxed 1.5 hr under nitrogen with 1 *N* KOH in 500 ml 95% ethanol (10). Unsaponifiables (0.79 g) were extracted with ether-hexane (1:1). Soaps were acidified (pH 2) with 6 *N* HCl and extracted with ether-hexane (1:1) to yield 16.7 g free fatty acids (FFA).

Esterification

The nonpolar FFA obtained from column chromatography (9) were esterified with 4% HCl in methanol by refluxing 3 hr under nitrogen.

Chromatographic Methods

Column chromatography: Separation of nonpolar fatty acid methyl esters (FAME) was achieved with 100 g 60-200 mesh HiFlosil-Ag (20% $AgNO_3$ on silica) (Applied Science, State College, PA) in a 3.5 cm ID x 58 cm glass column (11). To the column was applied 1.6 g isano FAME in hexane; the column was eluted consecutively with 750 ml of hexane-benzene (85:15), 500 ml benzene, and 500 ml benzene-ether (1:1). The column was regenerated with hexane-benzene (85:15) and more FAME were applied until six 1.6-g batches were separated.

Gas liquid chromatography (GLC): An F&M model 402 instrument without an injection heater and with dual flame ionization detectors

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at 250 C was used with the following columns: (a) a 0.6-cm (OD) by 122-cm glass column packed with 5% Apiezon L on 60/80 mesh acid washed (AW) Chromosorb W treated with dimethyldichlorosilane (DMCS), and (b) a 0.6-cm (OD) by 366-cm glass column packed with 7% LAC-2-R-446 on 60/100 mesh AW-DMCS Chromosorb W. The columns were operated isothermally at 190 C and samples were injected directly on the columns. The GLC results are expressed in equivalent chain length (ECL) values with the figure for ApL first, followed by R-446. A Bendix model 2600 instrument also was used with flame ionization detector at 350 C and injector temperature of 230 C; it was equipped with a 0.3-cm (ID) by 105-cm stainless steel column packed with 3% Silar 5CP on 80/100 mesh Gaschrom Q and was temperature programmed from 130 to 250 C at 4 C/min.

Thin layer chromatography (TLC): Commercial silica gel plates containing F-254 dye (Merck, Darmstadt, Germany) were used as received or were modified chemically for special applications. Components were visualized under short UV light (254 nm), by spraying with 0.2% ethanolic 2',7' dichlorofluorescein, by exposure to iodine vapor, by charring with 3% $K_2Cr_2O_7$ in 80% H_2SO_4 at 130 C, or by a combination of these methods. FAME were examined on argentation plates prepared by dipping the commercial 0.25-mm layer plates into a solution of 5% $AgNO_3$ in ethanol-water (60:40) and air drying before use. Development occurred in benzene-chloroform (85:15). The nonpolar FAME were also examined on plain silica gel plates developed in hexane-diethyl ether (60:40).

Preparative TLC: Commercial preparative plates (2-mm layers) were dipped into 5% $AgNO_3$, air dried, and used to separate fraction 4 (from column chromatography). Triple development in hexane-benzene (80:40) separated fraction 4 into two fractions: 4-1 (monoene FAME) and 4-2 (diyne FAME). Untreated preparative plates were used to separate fraction 5-4 (see below). These plates were subjected to multiple development with hexane-diethyl ether (70:30) consecutively the following distances: 1, 7.5 cm; 2, 10 cm; 3, 12.5 cm; 4, 15.0 cm. Separated components were located under short UV light.

Countercurrent Fractionation

Fraction 5 (mostly vinyl FAME) was subjected to countercurrent distribution (CCD) in a 200 tube Craig-Post apparatus with the solvent system acetonitrile-hexane (12). Throughout the distribution, 10 ml of upper phase and 40 ml lower phase were used. After 200 transfers, the upper phase was collected, three transfers per tube, in the fraction collector until 1400 transfers had occurred. Solvent was removed from the samples with a rotating evaporator.

Spectrometric Methods

IR: Spectra were obtained on 1% CCl_4 solutions with a Perkin-Elmer model 137 or model 700 spectrometer.

UV: Spectra were obtained on 0.1% methanol or cyclohexane solutions with a Beckman model DK-2A spectrophotometer.

*Proton Magnetic resonance (PMR):*Spectra were recorded with a Varian HA-100 instrument on samples dissolved in deuterochloroform with tetramethylsilane (TMS) as the internal standard.

Carbon-13 magnetic resonance (CMR): Spectra were determined on a Bruker WH-90 Fourier Transform NMR spectrometer, operating at 22.63 MHz with proton noise decoupling. The computer date memory size used for the real part of the spectra was 4K. The δ values are accurate to within ± 0.1 ppm. A 5 μsec (ca. 30°) pulse width was used. The spectra (5000-50,000 accumulations) were obtained from solutions in $CDCl_3$ which also served as an internal deuterium lock. Chemical shifts are given as δ-values in ppm downfield from the internal TMS-^{13}C signal.

Gas Chromatography-Mass Spectrometry (GC-MS): Spectra were obtained with a Beckman model 2600 gas chromatograph and a DuPont (CEC) 21-492-1 mass spectrometer using the procedure of Kleiman and Spencer (13).

RESULTS

Column Chromatography

Silica column chromatography (9) of the mixed isano acids yielded 66% nonpolar fatty acids. These were converted to methyl esters which were separated according to the accompanying scheme (Fig. 1), in which yields and R_f values are given.

Fraction 1

This liquid fraction is an unidentified substance whose IR suggest a hydrocarbon. It migrates to the solvent front on TLC and is unstable to GLC. The UV maxima are given in Table I. This fraction represents 1.8% by weight of isano oil (Table II).

Fraction 2

As analyzed by GLC, this fraction has 47%

16:0, 37% 18:0, 5% 20:0, 5% of several other saturated FAME and 6% of unidentified components. The amounts of these components present in isano oil are shown in Table II.

Fraction 3

By GLC, this fraction contains 83% of a component with ECL 19.6 (24.2), most of the remainder being saturated or olefinic C_{16} and C_{18} esters. The IR indicates conjugated diyne (C≡C stretch at 2247 and 2174 cm^{-1}) and ester carbonyl (1748 cm^{-1}) with no vinyl or *trans* double bond absorption. The UV absorption was more intense than that of the original mixture (Table I). PMR has δ0.88 (3H, t, J = 7 Hz), 1.32 (18H, m), 2.22 (4H, t, J = 6 Hz), 2.28 (2H, t, J = 6 Hz), and 3.63 (3H, s); these values are similar to those for a 10,12-diyne with 18 carbons (16). Also present in the PMR spectrum are weak multiplets which are indicative of a *cis*-double bond adjacent to a triple bond as in the following system:

$$\overset{c}{-CH_2}-\overset{a}{CH}=\overset{b}{CH}-(C\equiv C)_n-\overset{d}{CH_2}-$$

From this partial structure, the following assignments follow (17,18): a, δ6.02 (J_{ab} = 11 Hz, J_{ac} = 7 Hz); b, δ5.42 (J_{ab} = Hz, J_{bd} = ca. 1 Hz). These signals are not fully developed in intensity and suggest an impurity at the 25-30% level.

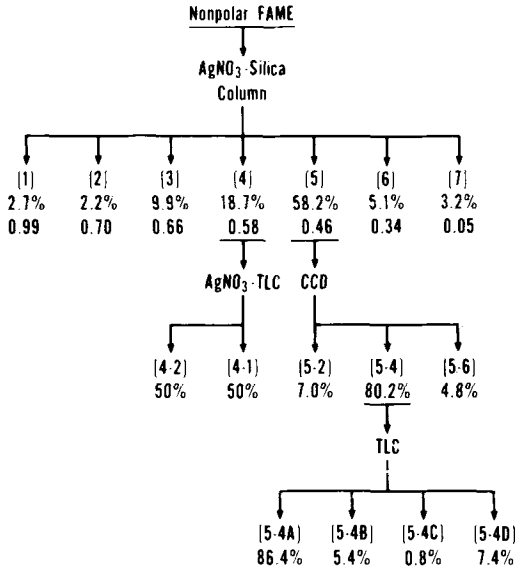

FIG. 1. Separation of the nonpolar fatty acid methyl esters (FAME) of Isano Oil. [Fraction number is listed first, followed by weight percent and the R_f value on argentation thin layer chromatography using benzene-chloroform (85-15)].

GC-MS of fraction 3 on Silar 5 CP gave one peak that shows two M^+ (*m/e* 288 and 290)

TABLE I

UV Absorption of Nonpolar Fatty Acid Methyl Esters (FAME) of Isano Oil (Expressed as $E_1^{1\%}$ cm)

Fraction	λ_{max} (nm)[a] 214 ± 1	228 ± 1	240 ± 1	254 ± 1	268 ± 1	283 ± 1	%[b] Enediyne	%[c] Diynone
1[d]	73(+)	---	---	15(+)	---	---		
2[d]	58	inf.	---	17	12	8	1.6	5.1
3	570	91	106	127	182	147	24.0	77.4
4-1	---	18	---	---	---	---		
4-2	682(+)	122	122	130(-)	176	142	23.2	74.9
5	47	---	28	30	32	25	4.2	13.6
5-2	---	11(+)	---	---	---	---		
5-4	100	30(-)	32	38	43	34	5.7	18.3
5-4A	106	22(-)	26	32	37	30	4.9	15.7
5-4B[e]	---	81(+)	96(+)	135	190	145	25.1	80.8
5-4C	---	99(+)	96	79	81	72	10.7	34.4
5-4D	---	32(+)	32(+)	34	39	31	5.1	16.6
5-6	---	---	---	62	58	45	7.7	24.7
6	---	100	---	---	42	34	5.5	17.9
7	134	inf.	inf.(-)	83(-)	80	59	10.6	34.0

[a]The parenthetical signs after E values signify higher (+) or lower (-) wavelength than given in heading; inf. = inflection, --- = not detected.

[b]Calculated as methyl octadec-*cis*-13-ene-9,11-diynoate and based on ε = 21,800 at 267.5 nm for the synthetic acid (14).

[c]Calculated as methyl 8-oxo-octadec-17-ene-9,11-diynoate and based on ε = 7100 at 267 nm for 10-hydroxy-4,6-decadiyn-3-one (15).

[d]Also has inflection at 327 nm.

[e]Also has E = 15 at 315 nm.

TABLE II

Nonpolar Components of Isano Oil
(% by wt)

Component[a]	Nonpolar fraction[b]	Oil[c]	Literature[d]
Unidentified hydrocarbon	2.7	1.8	--
16:0	1.5	1.0	4
16:1	0.2	0.1	--
18:0	1.2	0.8	1
18:1	13.3	8.8	14
18:2	0.3	0.2	5
18:3	3.5	2.3	--
20:0	0.2	0.1	--
20:1	0.2	0.1	--
9a,11a-C_{18}	9.7[e]	6.4[e]	10
9a,11a,17e-C_{18} (isanic acid)	45.6	30.1	32
8 oxo,9a,11a,17e-C_{18}	2.6	1.7	--
Unidentified C_{18} acetylenic acids	5.8	3.8	(9)[f]
Unidentified components	13.1	8.7	--
	99.9	65.9	75

[a]Number of carbons:number of double bonds; a = acetylenic bond, e = ethylenic bond of unspecified configuration.

[b]Based on gas liquid chromatography of the various fractions obtained from countercurrent distribution column and thin layer chromatography, except for the unidentified hydrocarbon which is weight %.

[c]Calculated back to original oil, 66% of which was in nonpolar fraction. The polar fraction was reported in (9).

[d]References (2) and (3) based on original oil; these also mention 1% 14:0.

[e]Includes any unresolved conjugated *cis* enediyne that may be present.

[f]Reported as 1% 9a,11e-C_{18}; 2% 9a,11a,13c-C_{18}; and 6% 9a,11a,13c, 17e-C_{18}; where c = *cis* ethylenic bond.

TABLE III

Gas Chromatography-Mass Spectrometry Data for Fractions 3, 4-2 Gas Liquid Chromatography Peak 6, and 5-4

	Relative intensity				Relative intensity		
m/e	3	4-2 Peak 6	5-4	*m/e*	3	4-2 Peak 6	5-4
39	27	100	33	131	40	55	93
41	88	88	93	133	28	53	21
43	37	54	21	145	24	39	63
55	87	76	58	146	37	37	45
59	26	42	49	148	12	25	3
67	54	70	56	159	9	17	18
79	56	63	51	160	4	9	6
81	30	50	48	162	5	14	0.5
91	100	99	100	173	6	11	18
92	30	56	25	187	6	9	9
93	36	62	33	220	2	6	---
105	58	82	68	234	1	3	---
106	21	50	17	257(M_1-31)	1	0.5	1
107	20	35	10	259(M_2-31)	5	10	---
117	48	60	72	288(M_1^+)	0.8	0.6	0.7
119	59	84	30	290(M_2^+)	0.8	1	---

and two M - 31 ions (Table III) indicative of C_{18} methyl esters, one with diyne unsaturation, and another with an enediyne system. MS of polyacetylenic compounds contain several common fragments, such as C_9H_9 (*m/e* 117) and C_7H_7 (m/e 91) (17), and they are observed in the MS of several of our fractions.

Fraction 4

This fraction was a mixture (as shown by argentation TLC) and was separated by preparative argentation TLC into two fractions (Fig. 1).

Fraction 4-1

This fraction was 97% methyl oleate by GLC, and the remainder was 16:1 and 20:1.

Fraction 4-2

The IR of this fraction has a very weak C≡C

stretch (2242 and 2174 cm^{-1}). The UV absorption is enhanced as in fraction 3 (Table I). PMR of this fraction is very similar to that of fraction 3, including weak signals that suggest a conjugated *cis*-eneyne structure. GC-MS of fraction 4-2 on Silar 5 CP gave several peaks; the MS of four of them are included in Tables III, IV, and V. Peak 2 (16% by GLC) gave fragments(*m/e* 152, 164, 166, 178, 196, and 210) suggesting methyl stearolate as its identity (19). Peak 3 (4%) and Peak 4 (9%) gave fragments (*m/e* 79, 93, 150, and 164) indicative of C_{18} eneyne structure with the triple bond between C-9 and C-10 (19). The ECLs of these two peaks suggest that the unsaturation of Peak 3 is not conjugated, whereas the unsaturation of Peak 4 would be conjugated (20). Peak 6 (64%) gave two M^+ and M-31 ions, like the MS of fraction 3 (Table III), indicating a mixture of C_{18} esters containing diyne and enediyne unsaturation that were not separated by GLC.

TABLE IV

Gas Chromatography-Mass Spectrometry Data for Fraction 4-2 Gas Liquid Chromatography Peak 2

m/e	Relative intensity
39	100
41	71
55	68
67	78
68	62
81	72
95	54
109	30
136	20
152	16
164	18
166	6
178	15
196	5
210	7
220(M-74)	2
263(M-31)	2
294(M^+)	1

Fraction 5

This fraction was the largest obtained by column separation (Fig. 1); its IR indicated that a vinyl group was present and the typical UV maxima were observed (Table I). Fraction 5 was separated by CCD and on the basis of its weight curve, three subfractions were examined further.

Fraction 5-2

This fraction represented CCD transfers 260-382 and contained 7% of the sample. GLC indicated that this fraction was 96% methyl oleate.

Fraction 5-4

This fraction contained 80% of the sample fractionated by CCD and comprised tubes 119-199 of the fundamental series. The major peak by GLC has ECL 19.7 (25.1). GC-MS gave M^+ and other ions compatible with methyl isanate (Table III). TLC on silica gel suggested there was some diynone present, so it was separated by preparative TLC. Four subfractions were obtained (Fig. 1).

Fraction 5-4A: There was 97% of one component with ECLs of 19.6 and 24.8 by GLC. The IR of this fraction indicated vinyl (3080, 1635, 990, and 910 cm^{-1}), C≡C stretch (2170 cm^{-1}), and ester carbonyl (1730 cm^{-1}). The UV maxima are given in Table I. The previously assigned structure of isanic acid (21) was supported by both PMR and CMR (22-25) (Fig. 2).

TABLE V

Gas Chromatography-Mass Spectrometry Data for Fraction 4-2 Gas Liquid Chromatography Peaks 3 and 4

	Relative intensity	
m/e	Peak 3	Peak 4
39	83	66
41	48	42
55	41	41
67	44	49
79	100	100
80	79	86
93	46	52
107	23	28
121	15	18
135	12	11
150	51	57
164	3	10
261(M-31)	1	2
292(M^+)	5	2

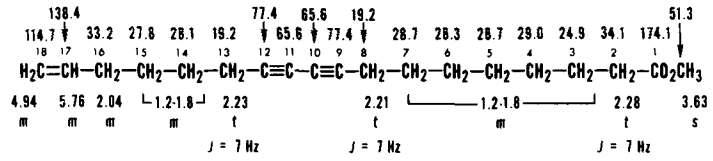

FIG. 2. Carbon-13 magnetic resonance (CMR) and proton magnetic resonance (PMR) of methyl isanate. The chemical shifts (δ) above the structure are for CMR, whereas PMR shifts are given below with multiplicity and coupling constants.

The amount of isanic acid present in isano oil is 30% (Table II).

Fraction 5-4B: The IR, UV, and TLC characteristics of this fraction indicated that methyl 8-oxo-octadec-17-ene-9,11-diynoate was present (9). The amount present according to UV absorption was 81% (Table I). GLC, however, showed that this fraction contained at least four different compounds.

Fraction 5-4C: This was the smallest fraction (<1% by weight) (Fig. 1) obtained from fraction 5-4. IR, UV, and TLC indicated that diynone (34% by UV, Table I) was present.

Fraction 5-4D: IR of fraction 5-4D indicates the presence of OH (3510 cm^{-1}), C≡C stretch (2260, 2240, and 2150 cm^{-1}), two carbonyls (1730 and 1675 cm^{-1}), and some vinyl absorption (weak bands at 3080, 1640, 990, and 910 cm^{-1}). This fraction was a mixture by TLC and GLC. The quantity isolated did not permit further characterization.

Fraction 5-6

This fraction represented tubes 9-49 in the fundamental series of CCD and amounted to 5% of the total weight (Fig. 1). The IR of this fraction indicates that FFA are present. UV is given in Table I. GLC on ApL and Silar 5 CP columns shows about 75% of the sample is in four unidentified peaks. Most of the sample remains at the origin in TLC on plain silica gel.

Fraction 6

Results by GLC indicate about 70% C_{18} triene (nonconjugated) as the major peak; this observation was supported by TLC on both plain and silver nitrate-impregnated silica gel plates. However, IR shows a small amount of vinyl and C≡C stretch due to overlap or tailing during the column separation. Other components of uncertain identity are present according to GLC.

Fraction 7

This fraction is a mixture of many components as shown by TLC and GLC. The IR suggests that it contains FFA, and UV has maxima indicative of the enediyne or diynone chromophore (Table I).

DISCUSSION

The experimental results described in this paper provided additional evidence which supports the occurrence in isano oil of constituents with conjugated *cis*-enediyne groupings. However, we have not proven their existence by isolating them in pure form.

The chromatographic separation scheme reported in our preceding paper (9) was intended to separate isano methyl esters into hydroxy and nonhydroxy ester fractions on plain silica. A second stage of column separation, described in the present paper and based on argentation chromatography, was intended to segregate esters with terminal double bonds from the rest. These column procedures were patterned after those used with *Acanthosyris* esters (10), which are similar in many respects to isano esters. However, the occurrence of keto acids in isano oil was not anticipated; because of their intermediate polarity, the conjugated diynone esters appeared in both "hydroxy" (polar) and "nonhydroxy" (nonpolar) fractions. The nature of their UV spectra is such that they are responsible for part of the absorption in both polar (9) and nonpolar fractions previously attributed to the *cis*-enediyne chromophore.

Of the fractions initially produced by argentation chromatography (Fig. 1), the most intense UV absorption that might be associated with the *cis*-enediyne chromophore was concentrated initially in Fractions 3, 4, and 5. By preparative argentation chromatography, fraction 4 was resolved into two subfractions, one of which (4-2) showed enrichment of the enediyne chromophore. Chromatographic fraction 5 was divided by CCD into three subfractions, one of which (5-4) had elevated UV absorbance; this was further resolved into four subfractions by preparative TLC. This TLC separation was difficult and was complicated by overlap; the highest UV absorbance was in subfraction 5-4B, and this proved to be due primarily to a conjugated diynone.

In contrast, fractions 3 and 4-2 gave PMR and mass spectra which were consistent with conjugated *cis*-enediyne structures. As described under Results, their PMR spectra were in accord with a double bond adjacent to a triple bond or a conjugated triple bond system (17). The observed coupling for the two olefinic protons (J = 11 Hz) indicates a *cis* configuration (18). In the MS of fractions 3 and 4-2, ions appear at m/e 288, 2 amu less than M^+ for the diynes which predominates in these fractions. This PMR and MS evidence lends some support to the hypothesis, previously based almost entirely on UV spectra, that isano oil contains acid with a *cis*-enediyne grouping. However, we were unable to resolve such substances from corresponding diynes so as to detect them by TLC with either plain silica or argentation systems.

We found PMR and MS evidence favoring the existence of the conjugated *cis*-enediyne grouping only in fractions without the terminal

double bond, not in corresponding fractions that do incorporate terminal double bonds. GLC analyses indicate the presence of minor amounts (<1%) of numerous components that remain unidentified.

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Effects of a Single Ingestion of Sodium Taurocholate on Esterified Cholesterol Concentration in Liver and Cholesterol Turnover in the Rat

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ABSTRACT

The overall response of the rat's cholesterol metabolism to a single ingestion of taurocholate (80 mg) was studied with the isotopic equilibrium method. The bile acid production, measured by the daily $^{14}CO_2$ output of rats in isotopic equilibrium of [26-^{14}C]-cholesterol, initially decreased and then increased. Conversely, the hepatic concentration of esterified cholesterol first increased and then decreased. Moreover, the ingestion of taurocholate increasing the intestinal absorption coefficient of dietary cholesterol increased the abosprtion and decreased the fecal excretion and the intestinal biosynthesis of cholesterol. The balance of these last effects is an excess cholesterol inflow. The classical hypothesis of negative feedback regulation of bile acid production fails to explain the observed biphasic effect of taurocholate. This compound, when its origin is exogenous, appears to stimulate the storage of esterified cholesterol in the liver, at the expense of bile acid synthesis. This accumulation rate takes into account not only the decrease in cholesterol transformation into bile acids but also the excess inflow of cholesterol. As the exogenous taurocholate was eliminated from the body, cholesteryl ester hydrolysis occurred and provided a supplementary source of free cholesterol for bile acid synthesis.

INTRODUCTION

The feedback regulation theory for bile acid production has been established from the following observations. Interruption or reduction of the enterohepatic circulation of bile acids by bile fistula, bile duct ligation or cholestyramine feeding increases the cholesterol biosynthesis (1), the cholesterol 7α-hydroxylase activity, and the bile acid production (2). Intraduodenal infusion of sodium taurocholate to bile fistula rats decreases this production (3). The mechanism of this effect might involve one or more processes. In rats, taurocholate or taurodeoxycholate reduces the activity of both cholesterol 7α-hydroxylase and HMG CoA reductase, although taurochenodeoxycholate acts only on the reductase (4). For others, bile acids increase the flow of cholesterol to the liver which, in turn, decreases the cholesterol and bile acids biosynthesis (5).

In a previous study (6), we showed that continuous taurocholate feeding causes a limited increase in hepatic esterified cholesterol, accompanied by an initial decrease in cholesterol transformation into bile acids and followed by a return to normal values. This suggests that exogenous taurocholate stimulates accumulation of esterified cholesterol in liver and that this accumulation secondarily influences bile acid production. To confirm this mechanism, we studied the effects of a single taurocholate ingestion. In a preliminary report, we showed that a single ingestion of various bile acids induced biphasic variations of bile acid production (7). In the present study, the overall response of the rat cholesterol system is analyzed. This implies the determination of the rates of all cholesterol turnover processes and of the size of the various cholesterol pools.

METHODS

Male adult rats of the Wistar Strain, weighing ca. 400 g, were kept under controlled light schedule (8). Each day at 17.00 hr the rats received 16 g of a semipurified diet (9) containing 0.05% of cholesterol. The isotopic equilibrium method, the theory and applicability of which have been described earlier (10,11), was used in a first experiment to estimate the bile acid production and in a second one to permit the simultaneous measurement of absorption, excretion, synthesis, and transformation of cholesterol.

In Vivo Measurement of Bile Acid Production

Three rats were placed in individual metabolism cages connected to an apparatus which permitted the measurement of expired $^{14}CO_2$ (12). The diet contained [26-^{14}C]-cholesterol

(30 μCi/100 g purchased from C.E.A., Saclay, France). Radioactivity of CO_2 was monitored continuously, and the integration of the curve over 1 hr or 1 day permitted calculation of hourly or daily $^{14}CO_2$ output. When an isotopic equilibrium was reached, i.e., when the daily output of $^{14}CO_2$ remained constant, the ratio of total radioactivity of expired CO_2 over 24 hr to the specific activity of hepatic free cholesterol, which is the direct precursor of bile acids (13), defined the amount of cholesterol transformed into bile acids per day (14). The experiment was started when the rats were in isotopic equilibrium of [26-^{14}C]-cholesterol, after about 30 days of radioactive diet. The first day the rats received 80 mg of taurocholate (purchased from Calibiochem, Eurobio, Paris, France) mixed in their diet.

Cholesterol Levels, and Rats of Cholesterol Turnover Processes

Two isotopic equilibria were performed on the same animals by subcutaneous injections ([^{3}H]-cholesterol–0.2 mg–1 μCi/day per rat from C.E.A.) and orally ([4-^{14}C]-cholesterol from C.E.A., 0.067 μCi/day per rat). Taurocholate feeding (80 mg) occurred 45 days after the beginning of isotope administration. The rats were killed at 10.00 hr, by intraaortic puncture, 1, 2, 5, 8, and 12 days after the taurocholate ingestion. The cardiovascular system was rinsed 3 times with saline. Livers were collected and washed again by the portal vein. Heparinized blood was centrifuged (2,200 g–4 C for 20 min) and plasma and red cells were recovered.

Free and esterified fractions of plasma and liver cholesterol were separated by silicic acid column chromatography (15). After saponification and extraction, cholesterol was precipitated by digitonin and measured by the Lieberman-Burchard reaction (15). Feces were collected daily and analyzed for sterol and bile acid contents. Feces were ground, extracted for 48 hr by ethanol-water (80:20) and saponified (ethanol, 2N KOH for 3 hr). Sterols were extracted by petroleum ether, precipitated by digitonin, and analyzed by gas liquid chromatography (GLC) (QF 1–1%) (16). The radioactivities were measured in a liquid scintillation spectrometer (Intertechniques Plaisir, France). The cholesterol absorption, internal and external secretion, excretion and transformation into bile acids rates were calculated, using the specific activities of mobile cholesterol (mean value of cholesterol specific activities in plasma and liver) or of dietary cholesterol and the radioactivities of neutral and acidic fecal steroids (11). The rates of cholesterol turnover

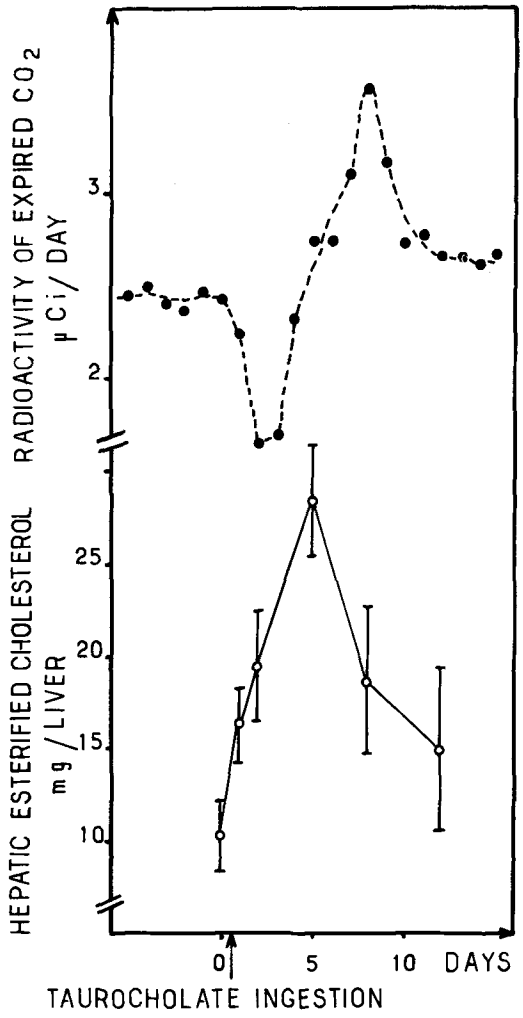

FIG. 1. Variations in the daily $^{14}CO_2$ output of a rat, initially in isotopic equilibrium of [26-^{14}C]-cholesterol and changes in hepatic esterified cholesterol quantities after a single ingestion of taurocholate (80 mg).

were determined on groups of 4 rats and hepatic cholesterol levels on groups of 9 rats. Results were compared to those of control rats being under the same experimental conditions but not fed on taurocholate.

Distribution and Elimination of Exogenous Taurocholate Kinetics

The rats received at 17.00 hr in their diet, 80 mg of [24-^{14}C]-taurocholate (1 μCi from N.E.N. Chemicals, GmbH). In a first experiment, the animals were killed by groups of 3, at 19.00, 22.00, 01.00, 04.00, 07.00, 10.00, and 17.00 hr. Radioactivities of contents and walls of the digestive tract and of the liver were meas-

TABLE I

[4-^{14}C]-Cholesterol Specific Activities (dpm/mg) in Red Cells, as Free (F) and Esterified (E) Fractions in Plasma and Liver of Rats in Isotopic Equilibrium, after a Single Taurocholate Ingestion (80 mg)

		Days after taurocholate ingestion					
		0	1	2	5	8	12
Red cells		4676 ± 200[a]	4752 ± 305	4706 ± 396	5257 ± 136	4614 ± 151	4827 ± 281
Plasma	F	4679[b]	4832	4889	5362	4538	5247
	E	4612	4615	4764	5296	4410	5085
Liver	F	4691	4765	4758	5202	4497	4714
	E	4979	5042	4724	5225	4497	4958

[a]Mean ± SEM (4 rats)

[b]Mean value of 4 rats.

TABLE II

Cholesterol Concentration (mg/100 g) as Free (F) and Esterified (E) Fractions in Plasma and Liver of Rats after a Single Taurocholate Ingestion (80 mg)

		Days after taurocholate ingestion					
		0	1	2	5	8	12
Plasma[a]	F	23	22	23	22	22	20
	E	65	63	66	68	64	59
Liver[b]	F	158 ± 4	172 ± 5[c]	167 ± 5	177 ± 4[c]	157 ± 9	155 ± 15
	E	63 ± 12	95 ± 13	120 ± 20[c]	168 ± 33[d]	111 ± 24[c]	92 ± 27

[a]Mean value of 4 rats.

[b]Mean ± SEM (9 rats).

[c]$P < 0.05$

[d]$P < 0.001$.

ured. In a second experiment, the feces of 4 rats were collected daily for 16 days and radioactivity measured. The time course of the fecal elimination was analyzed following the Linstedt and Norman method (17).

RESULTS

Taurocholate feeding first decreased (during 4 days) and then increased (during 6 days) the $^{14}CO_2$ output of rats in isotopic equilibrium of [26-^{14}C]-cholesterol (Fig. 1). Maximal decrease (32%) was observed in the second and the third day of the experiment. During all these changes in $^{14}CO_2$ production, the circadian variations of the $^{14}CO_2$ elimination (7,14) were maintained. These variations in daily $^{14}CO_2$ output reflect effectively changes in hepatic bile acid synthesis because the specific activity of their precursor (hepatic free cholesterol) remained constant during all the experiment as shown by the results obtained on rats fed [4-^{14}C]-cholesterol (Table I).

The ingestion of 80 mg of taurocholate did not modify the concentrations of free and esterified fractions of plasma cholesterol (Table II). The free cholesterol of liver changed slightly. But taurocholate feeding increased strikingly the hepatic concentration of esterified cholesterol (Table II). After 5 days, the quantity of esterified cholesterol in the liver was 28.3 mg, which represented a 18.1 mg increase as compared with the control value (Fig. 1). After 5 days, the hepatic concentration of esterified cholesterol decreased nearly to the control levels (Fig. 1, Table II).

Taurocholate feeding increased the intestinal absorption of dietary cholesterol from 65.2 to 90% (Fig. 2). This increase occurred the second day of the experiment and lasted 3 days. Then the intestinal absorption of cholesterol came back progressively to the control value. The increase in the absorption coefficient was accompanied by a marked decrease in the fecal elimination of sterol which can be explained by the reduction of the rates of excretion, external secretion, and nonabsorption of cholesterol (Fig. 2). Moreover, the fecal elimination of bile acids, was first lowered and then enhanced (Fig. 2) as it was observed in the first experiment for the $^{14}CO_2$ output.

The ingestion of [24-^{14}C]-taurocholate began at 17.00 hr. After only 2 hr the liver contained 0.5% of the total dose and 2.5%

(maximal value) 11 hr after the beginning of experiment. The radioactivity of the intestine (content + wall) was maximal after 8 hr of experiment and represented 50% of the ingested dose. The first day of the experiment (17.00-10.00 hr) 4% of the radioactive ingested dose was recovered in the feces, but during the following days, the taurocholate was quickly eliminated ($t_{1/2}$ = 0.64 day) and 75% of the dose was removed after 5 days (Fig. 2). Then, a second period of elimination began ($t_{1/2}$ = 2.7 days) corresponding to the fecal elimination of cholic acid (17).

DISCUSSION

The absorption coefficient of dietary cholesterol, calculated from radioactivities measured in the feces collected during the first day of experiment, was unchanged (65.7% vs. 65.2%), but in the second day, this coefficient reached 90%. Taking into account the mean time of intestinal transit for sterols in the rat (18), the effects of taurocholate in the intestine could not be expected in the feces before the second day. Therefore, taurocholate effects at the intestine level appear to be immediate. The increased absorption was similar to that observed in continuous taurocholate fed rats (6). After five days of experiment, about 75% of the ingested taurocholate had been eliminated, and the cholesterol absorption began to fall. It has been previously shown that the higher the absorption coefficient of cholesterol, the lower the rates of cholesterol fecal excretion, the external secretion, and the total synthesis (19,20). The decreases of fecal excretion and external secretion which followed the taurocholate ingestion (Fig. 2) are only explained by the increased absorption coefficient. Therefore, taurocholate had no direct effect on these processes, in agreement with the previous results (6). The internal secretion rate (total synthesis minus external secretion) was 14.0 ± 0.6 mg/day for control rats. Slight and transitory variations of this process cannot be observed by the isotope dilution method. The mobile cholesterol pool in a rat [about 400 mg (21)] is such a large dilution factor for small radioactive variations that significant change in its specific activity could not be observed. We note that the internal secretion varied, like the other processes, as a consequence of changes in the absorption coefficient (19,20). This effect on cholesterol synthesis has been previously observed in rats chronically fed 0.2% of taurocholate (6). However, higher doses of taurocholate, 4% in the diet (6) or 100 mg/100 g body weight during 7 days (4), reduced

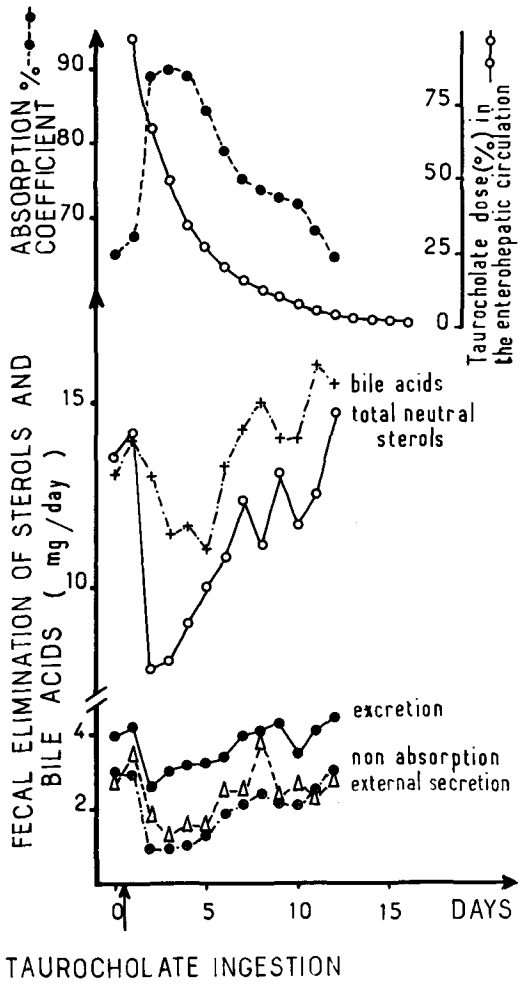

FIG. 2. Intestinal absorption coefficient of dietary cholesterol and fecal elimination of sterols, bile acids, and exogenous taurocholate in rats after taurocholate ingestion (80 mg). Data were calculated as follow (11): [^{3}H]-cholesterol and [4-^{14}C]-cholesterol were administrated respectively by subcutaneous injections and in the diet. Absorption coefficient: CA m_A/m_I. 100 with m_A: amount of dietary cholesterol absorbed; m_I: amount of dietary cholesterol ingested. Absorption: $m_A = m_I - m_{NA}$ with m_{NA}: amount of unabsorbed dietary cholesterol. Nonabsorption: $m_{NA} = R_{NA}/r_A$ with R_{NA}: total radioactivity of ^{14}C cholesterol unabsorbed, r_A: specific activity of dietary cholesterol. $R_{NA} = R_{F1} - R_{FE}$ with R_{F1}: ^{14}C radioactivity of fecal neutral sterol, R_{FE}: ^{14}C radioactivity of fecal excretion source. R_{FE} ^{14}C = $m_{FE} \cdot r_{P1}$ with r_{P1}: ^{14}C specific activity of mobile cholesterol, m_{FE}: fecal excretion rate. $m_{FE} = R_{F2}/r_{P2}$ with R_{F2}: ^{3}H radioactivity of fecal neutral sterol, r_{P2}: ^{3}H specific activity of mobile cholesterol. External secretion: $m_{ES} = m_F - m_{NA} - m_{FE}$ with m_F: total fecal cholesterol. $m_F = R_F/r_F$ with r_F: ^{3}H or ^{14}C radioactivity of fecal neutral sterol. r_F: ^{3}H of ^{14}C specific activity of fecal cholesterol. Transformation into bile acids: $m_T = R_{AC}/r_{P1}$ with R_{AC}: ^{14}C radioactivity of fecal acidic fraction.

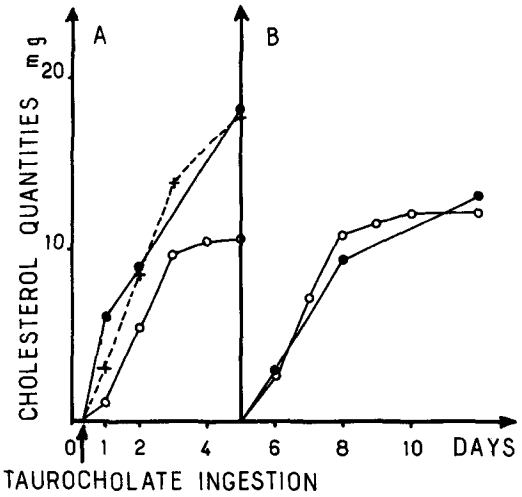

FIG. 3. Variation in the dynamic equilibrium of rat cholesterol after taurocholate ingestion (80 mg). (A) •–esterified cholesterol accumulation in liver; o–cummulative decrease in bile acid production; x–cummulative variations in the rates of cholesterol absorption, excretion, internal secretion, and transformation into bile acids. (B) •–esterified cholesterol elimination; o–cumulative increase in bile acid production.

strongly the hepatic (4) or the total synthesis of cholesterol (6).

The increase of absorption coefficient modified the steady state inducing a higher cholesterol absorption and lower excretion, and external and internal secretion rates. Considering these processes, the cholesterol balance was changed during this transitory period. Input exceeds output by 2.0 mg the first day and by 5.8 mg for the 4 following days. The results of such a balance could be an increased cholesterol transformation rate into bile acids. We have previously shown that any variation in the rates of excretion, internal secretion or absorption of cholesterol was accompanied by compensatory variation in the bile acid production rate, so that the cholesterol balance is maintained (6). This increase was not observed in the present study. After taurocholate feeding, 18 mg of esterified cholesterol were stored in the liver and the bile acid production was decreased (Fig. 1). This storage was of 6 mg per liver at the time of sacrifice the first day of experiment, i.e., 17 hr after the beginning of taurocholate ingestion, when the maximal effect on bile acid production was not yet obtained. The quantities of cholesterol accumulated were greater than the reduction in bile acid production by any time (Fig. 3A). Only the variations in the rates of fecal excretion, absorption, internal secretion, and transformation explain the quantity of cholesterol accumulated after 5 days of experiment (Fig. 3A).

The kinetic of the accumulation of esterified cholesterol and its magnitude corroborate our hypothesis concerning the primary action of exogenous taurocholate on the esterification-hydrolysis equilibrium of hepatic cholesterol, stimulating the esterification. The increase in cholesterol esterification, which explained the transitory decrease in bile acid formation during chronic ingestion of taurocholate (6), includes also the cholesterol provided by the excess flow reaching the liver. Moreover, quantities of cholesterol eliminated at 8 and 12 days of experiment correspond to the increase in bile acid production (Fig. 3B), the rate of the other processes of cholesterol turnover being similare to those of control rats. Hence, the increased rate of bile acid production, as the excess tuarocholate was eliminated from the body, is due to a supply of free cholesterol formed by the hydrolysis of hepatic cholesterol esters which were first accumulated. Biphasic variations of bile acid production induced by ethinyl estradiol treatment in rat have been similarly interpreted by Davis and Kern (22). Contrarily, the hepatic cholesterol storage observed by Shefer et al. in rats fed a higher taurocholate dose has been interpreted as a consequence of a reduction of the cholesterol 7α-hydroxylase activity (4).

In conclusion, exogenous taurocholate at a moderate level in the diet acts on the esterification-hydrolysis equilibrium of hepatic cholesterol, stimulating the storage of esterified cholesterol. The bile acid production is continuously adjusted in such a way that the law of conservation of mass is respected. In other words, its rate ensures a zero balance taking into account, not only all other flows of the cholesterol system, but also the changes of the cholesterol pool and particularly of hepatic esterified cholesterol. Nevertheless, the cholesterol 7α-hydroxylase, which is described as affected by bile acid feeding (4,23) or by the enterohepatic circulation of bile acids (24,25), could be an element of the adaptation of bile acid production.

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SHORT COMMUNICATIONS

Effects of Cyanide on Peanut Lipoxygenase

ABSTRACT

Acidic and alkaline lipoxygenase isozymes were separated and assayed for activity while incubated in the presence of various concentrations of cyanide. Inhibition due to cyanide per se was not found. Instead, cyanide caused an increase in pH, which decreased the activity of the acidic isozyme to a rate corresponding to the same pH on the activity curve for the cyanide-free reaction.

INTRODUCTION

In 1957, Siddiqi and Tappel (1) reported that cyanide did not influence the activity of peanut lipoxygenase when the enzyme was incubated at the usual inhibitor concentration of cyanide (1 mM). Using a partially purified enzyme preparation, St. Angelo and Ory (2) confirmed those results. Under different conditions, Sanders et al. (3) recently reported that cyanide inhibited a purified peanut lipoxygenase acid pH isozyme, but concentrations below 14 mM had little effect on the activity of the alkaline pH isozyme. Because of these conflicting reports, we reinvestigated the effect of cyanide on peanut lipoxygenase to reaffirm our earlier findings (2). Special attention was given to the effect of cyanide on pH of the reaction mixture before, during, and after measuring enzymic activity.

MATERIALS AND METHODS

Peanuts (*Arachis hypogaea L.*, var. VA. 56R) were obtained from a commercial supplier in Virginia; high purity linoleic acid from The Hormel Institute, Austin, MN; reagent grade potassium cyanide from Mallinckrodt, St. Louis, MO.

Peanut lipoxygenase isozymes were prepared using the extraction medium described by Sanders et al. (3). Raw peanuts were extracted with 0.5 M NaCl, 0.05 M Tris buffer, pH 7.2, and centrifuged twice at 29,000 g for 10 min at 0 C. A pH 6 isozyme was found in the resuspended precipitate; the alkaline isozyme appeared in the clear supernatant fraction. No attempt was made to separate the two acid isozymes.

Each isozyme was assayed for activity by the spectrophotometric method described previously (2), except that pH adjustments were made to 8.3 for the alkaline isozyme. Substrate solutions consisted of linoleic acid, Tween-20, in 0.1 M phosphate buffer as described by Ben-Aziz et al. (4). In each assay, the reaction mixture contained 1 ml of the linoleic acid substrate solution, 1.95 ml of 0.01 M phosphate buffer, and 0.05 ml of the enzyme preparation, all added to a 4 ml cuvette. When the effect of cyanide on peanut lipoxygenase was investi-

TABLE I

pH of KCN-Phosphate Buffer Solutions

Phosphate buffer (pH)	(M)	KCN (mM)	pH of final solution
6.1	0.01	30	9.5
6.1	0.10	30	6.8
6.1	0.10	300	9.8
8.3	0.01	30	10.8
8.3	0.10	30	9.7
5.7	0.20	300	8.9
4.7[a]	0.10	300	9.4
5.5	deionized water	30	10.5

[a] NaH_2PO_4.

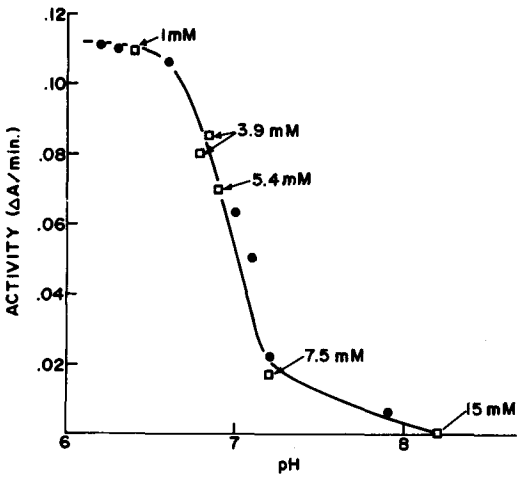

FIG. 1. Effect of cyanide on pH as related to lipoxygenase activity. Lipoxygenase activity was measured as the change in absorption/min at 234 nm. Circles represent the reaction rate from buffered enzyme and substrate; squares represent the reaction rate with cyanide added.

gated, a microvolume (25-100 μl) of cyanide solution was added to buffer containing the enzyme and allowed to stand for 5 min. Substrate was then added and the mixture assayed for lipoxygenase activity over the first 3 min. Immediately following the assay, the final pH of the reaction mixture was determined.

RESULTS AND DISCUSSION

Cyanides are poisons that combine with particular enzymes (usually those containing copper or iron) associated with cellular oxidation in living organisms. This reaction occurs because the cyanide can form very stable complexes with heavy metals. Although cyanide inhibits many enzymes that contain a metal as an essential part of the molecule, it also inhibits some enzymes that do not contain a metal prosthetic, group (5). Cyanide is also known to activate some plant proteases (6).

Since aqueous solutions of sodium or potassium cyanide are storngly alkaline, it was desirable to first determine the effect of cyanide concentration on the pH of buffered solutions of cyanide alone. Table I shows the pH of solutions containing KCN dissolved in phosphate buffers, one of the more popular biological buffers used. These results show that, as the concentration of cyanide is increased, even though it is added in dilute acidic buffers, the pH of the final solutions becomes very alkaline. Similar results were found when the experiments were repeated with the phosphate-borate buffer system used by Sanders, et al. (3). The KCN concentrations varied from 3.75 to 30 mM while the pH of the final solutions increased from 6.4 to 7.7.

At ordinary tempertures, aqueous solutions of sodium (or potassium) cyanide ionize according to the reversible reaction:

$$NaCN + H_2O \rightleftharpoons NaOH + HCN.$$

NaOH completely dissociates and functions as a strong base, whereas hydrocyanic acid is a realtively weak undissociated acid. The results observed in Table I and in the KCN-phosphate-borate buffer system indicate that caution is advisable when the pH of enzyme solutions is critical and show that the addition of cyanide can change the pH from the optimum for the enzyme under investigation. Such a difference in pH could lead to erroneous conclusions on the precise cause for loss of enzymic activity.

The results of further experiments with lipoxygenase and cyanide are presented in Figure 1. Without adding cyanide to the reaction mixture, and after adjusting the pH with appropriate phosphate buffer, a typical pH activity curve with an optimum activity plateau at 6.1 or 6.2 was observed. Very little activity occurred above pH 7. No experiments were conducted below pH 6.1 since the data confirmed previous reports of a pH optimum at 6.1 (2,3). When cyanide solutions were incubated with the enzyme-buffer prior to the addition of substrate, activity followed the same pH curve. At 1 mM cyanide, the pH of the reaction mixture was 6.2; with increasing concentrations of cyanide (3.9, 5.4, 7.5, and 15 mM), the pH changed to 6.8, 6.9, 7.2, and 8.2, respectively. This change indicated that increasing amounts of cyanide do not inhibit the pH 6.2 isozyme, but cause an increase in pH that suppresses the activity of the enzyme.

To confirm these results, an additional experiment was conducted on the effect of cyanide on lipoxygenase at optimum pH. A small volume of cyanide dissolved in 0.2 M monobasic sodium phosphate solution was added to combinations of 0.2 M phosphate buffers and incubated for 5 min with 50 μl of the acidic (pH 6.2) isozyme preparation in 2.0 ml total volume. The pH of the reaction mixture containing cyanide (15 mM) was still very near the optimum for lipoxygenase activity. After incubation, 1 ml of substrate solution was added as before, and the activities were measured. Results showed that the control sample, comprised of enzyme, substrate, and buffer at pH 6.1, had an initial activity of 0.074 (Δ A/min). The two samples containing the cyanide, one at pH 5.8, and the second at pH 6.2, had enzyme activities of 0.065 and 0.060, respectively. These results clearly indicate that high concentrations of cyanide dissolved in adequately buffered solutions at approximately the optimum pH, did not appreciably inhibit activity of the acidic isozymes.

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Effect of Tibric Acid on Hepatic Cholesterol Synthesis in Rats

ABSTRACT

Male albino rats were administered various oral doses of tibric acid daily for 1 week. Serum cholesterol and triglyceride levels were reduced, but total liver content of cholesterol, phospholipids, and triglycerides was increased. Tibric acid treatment suppressed the incorporation of both [^{14}C]acetate and [^{3}H]mevalonate into cholesterol by liver homogenates.

INTRODUCTION

Tibric acid {2-chloro-5-[(cis-3,5-dimethylpiperidino)sulfony]benzoic acid}, has been reported to be approximately 10 times more potent than clofibrate in lowering serum lipid levels in rats (1). The activity of tibric acid was ascribed to its capacity to increase the activity of liver mitochondrial α-glycerophosphate dehydrogenase, thus decreasing the rate of hepatic triglyceride synthesis (2). Kritchevsky et al. (3) have studied the catabolism of cholesterol in rats treated with tibric acid and found no change in the activity of hepatic 7α-hydroxylase or cholesterol-26-oxidase. Tibric acid was shown to be antihypertriglyceridemic in patients with Type IV hyperlipoproteinemia (4-7). Some investigators have shown that the drug lowers elevated cholesterol (6,8), while others have found no effect (4,7).

In order to elucidate further the mode of action of tibric acid, we have investigated the effect of the compound on hepatic cholesterol synthesis, and our findings are reported herewith.

METHODS AND MATERIALS

Tibric acid was kindly supplied through the courtesy of Dr. J.N. Pereira, Pfizer Central Research, Groton, CT. Male albino Sprague-Dawley rats, initially weighing 140-150 g, were kept under observation for 4 days and used only if food intake and body weight gain were normal. Tibric acid was suspended in 2% Tween-80 and administered p.o. by gastric intubation daily for 1 wk; ten rats per group were given 123 mg (400 nmole), 25 mg (80 nmole), or 13 mg (40 nmole)/kg/day of the compound, and were not fasted. Controls received 1 ml of 2% Tween-80 only. Animals were decapitated 3 hr after the last dose. Serum triglycerides were also measured in rats rendered hypertriglyceridemic by replacing the drinking water with a 10% fructose solution during the last 24 hr of treatment (9,10).

Serum and liver cholesterol levels were determined by the method of Zlatkis et al. (11), as modified for the autoanalyzer. Phospholipid and triglyceride levels were measured by semi-

TABLE I

Effect of Tibric Acid on Serum and Liver Lipids in Rats (Mean ± SEM for 10 rats/group)

Parameter	Controls	Tibric acid (125 mg/kg/day)	Tibric acid (25 mg/kg/day)	Tibric acid (13 mg/kg/day)
Body wt gain (g/wk)	50 ± 1.8	58 ± 3.1[a]	55 ± 1.7	59 ± 2.3[b]
Liver wt (g)	10.3 ± 0.19	16.2 ± 0.49[c]	14.3 ± 0.42[c]	13.2 ± 0.37[c]
Liver, % of body wt	4.5 ± 0.08	6.8 ± 0.21[c]	6.1 ± 0.16[c]	5.6 ± 0.14[c]
Cholesterol				
Serum (mg/dl)	73.8 ± 3.75	55.6 ± 3.26[b]	56.2 ± 2.49[b]	52.7 ± 1.89[c]
Liver (mg/100 g)	226 ± 4.6	210 ± 5.2[a]	201 ± 5.4[b]	189 ± 5.6[c]
Liver (mg/rat)	23.3 ± 0.41	34.0 ± 0.48[c]	28.7 ± 0.50[c]	24.9 ± 0.45
Phospholipids				
Serum (mg/dl)	125 ± 3.5	111 ± 5.5	112 ± 3.5[a]	110 ± 3.0[b]
Liver (mg/100 g)	3200 ± 50	3550 ± 35[c]	3400 ± 93	3350 ± 65
Liver (mg/rat)	330 ± 5.3	575 ± 6.5[c]	485 ± 10.3[c]	443 ± 6.3[c]
Triglycerides				
Serum, normal rats (mg/dl)	71.0 ± 7.00	40.5 ± 2.33[c]	41.8 ± 1.68[c]	50.3 ± 3.45[a]
Serum, hypertriglyceridemic rats (mg/dl)	192 ± 16.5	61 ± 4.2[c]	78 ± 11.4[c]	86 ± 8.4[c]
Liver (mg/100 g)	573 ± 28.2	642 ± 41.0	727 ± 47.2[a]	636 ± 22.5
Liver (mg/rat)	59 ± 3.4	104 ± 3.7[c]	104 ± 4.5[c]	84 ± 3.2[c]

[a]$p<0.05$.
[b]$p<0.01$
[c]$p<0.001$

TABLE II

Effect of Tibric Acid on the Incorporation of [^{14}C] Acetate and [^{3}H] Mevalonate into Cholesterol by Rat Liver Homogenates

	Dose or concentration	dpm Cholesterol/mg nitrogen	
		[^{14}C] Acetate	[^{3}H] Mevalonate
In vivo[a]	Controls	1850 ± 517	57,060 ± 5,320
	123 mg/kg/day	95 ± 28[e]	29,440 ± 4,060[d]
	25 mg/kg/day	42 ± 10[e]	21,720 ± 2,170[d]
	13 mg/kg/day	178 ± 41[e]	41,580 ± 3,710[c]
In vitro[b]	Controls	1430	59,700
	1 x 10^{-4}M	1510	58,200

[a]Rats were treated for 1 wk and liver homogenates were incubated simultaneously with 5 μCi [2-^{14}C] acetate, 1.3 μCi [2-^{3}H] mevalonate, and appropriate cofactors (15). Cholesterol was isolated, purified, and counted as its 5,6-dibromo derivative. Results are expressed as mean ± SEM for 9 rats/group.

[b]Tibric acid was dissolved in chloroform, and the solution was evaporated to dryness in 20 ml beakers. Rat liver homogenate, 2.4 μCi [2-^{14}C] acetate, 1.4 μCi [2-^{3}H] mevalonate, and appropriate cofactors were added, and the incorporation into cholesterol was measured. Previous results have shown that insoluble compounds added in this manner become suspended in the incubation medium and are thus capable of affecting cholesterogenesis. Data are averages of duplicate incubations.

[c]$P<0.05$.

[d]$P<0.01$.

[e]$P<0.001$.

automated procedures (12,13). Nitrogen levels were determined according to Ferrari (14). Changes in cholesterol synthesis were assessed by measuring the incorporation of [2-^{14}C] acetate (5 μCi) and [2-^{3}H] mevalonate (1.3 μCi) into cholesterol by liver homogenates (15) prepared from normal and tibric acid treated rats.

Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375.

RESULTS AND DISCUSSION

The effect of tibric acid on serum and liver lipid levels is summarized in Table I. The compound was markedly hepatomegalic after 1 wk of treatment (cf. 1,3). Dose-dependent decreases in serum triglycerides were observed in both normal and hypertriglyceridemic rats, confirming previous findings (1,3). Serum cholesterol was reduced at all dose levels. Kritchevsky et al. (3) found no change in serum cholesterol in rats fed 0.075% (ca. 75 mg/kg/day) of tibric acid for 3 wk; we did not determine whether the hypocholesterolemic effect of tibric acid can be reversed upon prolonged treatment. Serum phospholipids were virtually unchanged. In the liver, tibric acid produced a small decrease in cholesterol levels, but had no significant effect on the concentration of phospholipids and triglycerides. However, the pronounced hepatomegaly induced by tibric acid resulted in substantial increases in total liver lipid content, especially of triglycerides; this is in contrast to the report of Kritchevsky et al. (3).

The effect of tibric acid on the incorporation of [^{14}C] acetate and [^{3}H] mevalonate into cholesterol by rat liver homogenates is shown in Table II. Pretreatment of rats with tibric acid markedly inhibited cholesterol biosynthesis at sites both before and after the formation of mevalonate. The direct effect on cholesterol synthesis was determined in vitro, by adding tibric acid to a rat liver homogenate preparation containing [2-^{14}C] acetate and [2-^{3}H] mevalonate: at a final concentration of 1 x 10^{-4}M, tibric acid had no effect on the incorporation of either of the labeled precursors into cholesterol (Table II). Thus, tibric acid inhibits cholesterol biosynthesis in vivo, but not in vitro.

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Arylsulfonate Esters of Fatty Alcohols as Hypocholesterolemic Agents. I: Oleyl and Linoleyl p-Toluenesulfonates[1]

ABSTRACT

Cholesterol levels of both plasma and liver of cholesterol-fed rats are lowered by feeding linoleyl and oleyl esters of p-toluenesulfonic acid. A structural specificity exists, since the effect is not observed with similar amounts of sodium p-toluenesulfonate nor with linoleyl methanesulfonate. Effective levels of these arylsulfonates do not alter liver to body weight ratios and maturation, nor reduce plasma cholesterol in normocholesterolemic rats.

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INTRODUCTION

The mild action of polyunsaturated fats and their fatty acids in lowering blood cholesterol in some types of hypercholesterolemia is well known. When fed to experimentally hypercholesterolemic rats, linoleate of high purity not only lowers plasma cholesterol (1) but also tends to lower hepatic cholesterol and protect against atherosclerotic lesions (2); oleate has no hypocholesterolemic effect. These two fatty acids have now been reduced to the corresponding alcohols (3), esterified by reaction with p-toluenesulfonyl chloride (4), and the arylsulfonate esters have been tested for activity with hypercholesterolemic rats.

TABLE I

Response of Hypercholesterolemic Rats to Dietary Sulfonate Esters and Ethyl Esters in 11-Day Tests

Test substance	Level in diet A (%)	Number of rats	Gain, body weight (g)	Total cholesterol (mean ± SEM) Plasma (mg/100/ml)	Liver (mg/g)
Ethyl oleate	1.0	7	35	379 ± 19	40 ±2.8
Oleyl p-toluenesulfonate	0.5	4	42	126 ± 20[a,c]	29 ± 2.7[b]
Oleyl p-toluenesulfonate	0.1	4	24	171 ± 28[a]	36 ± 5.1
Sodium p-toluenesulfonate	0.46	4	6	334 ± 35	–
Ethyl linoleate	1.0	7	55	231 ± 25[b]	41± 2.9
Linoleyl p-toluenesulfonate	0.5	4	8	77 ± 11[a,c]	27± 1.7[b]

[a]Significantly different from ethyl oleate group ($P < 0.01$).
[b]Significantly different from ethyl oleate group ($P < 0.05$).
[c]Significantly different from ethyl linoleate group ($P < 0.01$).

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ABSTRACT

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INTRODUCTION

The mild action of polyunsaturated fats and their fatty acids in lowering blood cholesterol in some types of hypercholesterolemia is well known. When fed to experimentally hypercholesterolemic rats, linoleate of high purity not only lowers plasma cholesterol (1) but also tends to lower hepatic cholesterol and protect against atherosclerotic lesions (2); oleate has no hypocholesterolemic effect. These two fatty acids have now been reduced to the corresponding alcohols (3), esterified by reaction with p-toluenesulfonyl chloride (4), and the arylsulfonate esters have been tested for activity with hypercholesterolemic rats.

TABLE I

Response of Hypercholesterolemic Rats to Dietary Sulfonate Esters and Ethyl Esters in 11-Day Tests

Test substance	Level in diet A (%)	Number of rats	Gain, body weight (g)	Total cholesterol (mean ± SEM) Plasma (mg/100/ml)	Liver (mg/g)
Ethyl oleate	1.0	7	35	379 ± 19	40 ±2.8
Oleyl p-toluenesulfonate	0.5	4	42	126 ± 20[a,c]	29 ± 2.7[b]
Oleyl p-toluenesulfonate	0.1	4	24	171 ± 28[a]	36 ± 5.1
Sodium p-toluenesulfonate	0.46	4	6	334 ± 35	–
Ethyl linoleate	1.0	7	55	231 ± 25[b]	41± 2.9
Linoleyl p-toluenesulfonate	0.5	4	8	77 ± 11[a,c]	27± 1.7[b]

[a]Significantly different from ethyl oleate group ($P < 0.01$).
[b]Significantly different from ethyl oleate group ($P < 0.05$).
[c]Significantly different from ethyl linoleate group ($P < 0.01$).

TABLE II

Response of Hypercholesterolemic Rats to Dietary Sulfonates in 4-wk Tests

Test substance	Level in diet A (%)	Number of rats	Gain, body weight (g)	Total cholesterol (mean ± SEM) Plasma (mg/100/ml)	Liver (mg/g)
None–control	–	6	56	194 ± 21	43 ± 2
Linoleyl p-toluenesulfonate	0.05	5	56	135 ± 30	18 ± 1[a]
Linoleyl p-toluenesulfonate	0.15	6	52	65 ± 4[a]	15 ± 1[a]
Linoleyl methanesulfonate	0.15	6	8	243 ± 30	75 ± 10
Sodium p-toluenesulfonate	0.15	6	48	176 ± 15	62 ± 5

[a]Significantly different from the control group ($P < 0.001$).

TABLE III

Propylactic Effect of 0.15% Linoleyl p-Toluenesulfonate on Cholesterol-fed Rats in a 4-wk Period (7 rats per group)

Diet A Test substance	Gain, body wt (g/wk)	Liver wt Body wt	Cholesterol found (mean ± SEM) in Plasma Total (mg/100 ml)	Plasma Free (mg/100 ml)	Liver Total (mg/g)	Liver Free (mg/g)
None	23	0.066	273 ± 18	62 ± 4	35 ± 4	3.0 ± 0.1
Linoleyl p-toluenesulfonate	12	0.065	45 ± 6[a]	17 ± 1[a]	5 ± 1[a]	1.5 ± 0.9

[a]Significantly different from control group ($P < 0.01$).

TABLE IV

Propylactic Effect of 0.15% Linoleyl p-Toluenesulfonate on Weanling Female Rats Fed 20% Dietary Fat in Diet B During a 4-wk Period (six per group)

Dietary fat	LTS, %[a]	Gain, body wt (g/wk)	Total cholesterol (mean ± SEM) in Plasma (mg/100 ml)	Liver (mg/g)	Total lipid in liver (%)
Corn oil	none	22	186 ± 35	88 ± 6	24
Corn oil	0.15	21	110 ± 8	63 ± 4	20
Cottonseed oil	none	20	158 ± 35	74 ± 2	22
Cottonseed oil	0.15	21	115 ± 13	50 ± 1	17
H. coconut oil[b]	none	15	540 ± 91	36 ± 7	10
H. coconut oil	0.15	14	174 ± 13	14 ± 2	7

[a]Linoleyl p-toluenesulfonate.

[b]Hydrogenated coconut oil.

EXPERIMENTAL PROCEDURE

Two semisynthetic diets were used, one low in fat and the other high in fat, details of which have been described (1). Diet A was polyunsaturate-free. It contained 1% fat (hydrogenated coconut oil), 15% casein, 76% glucose, 1% cholesterol, and 0.5% sodium glycocholate. Diet B contained 20% fat, 18% casein, 55% glucose, 2% cholesterol, and 0.5% sodium glycocholate. To both diets vitamins and minerals were added in constant ratio to calories. Weanling albino rats of the Wistar strain were fed these diets ad libitum. Blood samples were obtained by heart puncture, and the cholesterol from both blood and liver was purified as the digitonide (5,6).

RESULTS AND DISCUSSION

Male rats that had consumed Diet A for 4 wk after weaning and were fed the arylsulfonates as an addition to the same diet, and the effects were then observed after 11 or 28 days. In 11-day tests (Table I), both oleyl p-toluenesulfonate (OTS) and linoleyl p-toluenesulfonate (LTS) were much more active as hypocholesterolemic agents than was ethyl linoleate. Both

sulfonate esters also reduced stores of hepatic cholesterol, whereas ethyl linoleate produced no measurable similar effect. In 28-day tests (Table II), linoleyl p-toluenesulfonate was highly effective in lowering cholesterol levels in both plasma and liver. Neither sodium p-toluenesulfonate nor linoleyl methanesulfonate showed ability to lower cholesterol. It appears, therefore, that activity is a function of both the alcohol moiety and the sulfonic moiety.

To observe whether a sulfonate ester could prevent cholesterol accumulation, as well as reduce levels which are already elevated, male weanling rats were fed LTS along with Diet A for a 4-wk period (Table III). Levels of free cholesterol as well as esterified cholesterol in both plasma and liver remained near the normal range (1) in the LTS-fed rats.

Since Diet A is deficient in the essential fatty acids (EFA), and a relationship is known to exist between EFA and cholesterol metabolism (7), an experiment was performed to observe the effects of LTS on animals consuming a high-cholesterol, high-fat diet (Diet B) in which corn and cottonseed oils supplied EFA in abundance (Table IV). High dietary levels of vegetable oils can promote hepatic deposition of dietary cholesterol in rats (1,8) despite the abundant presence of polyunsaturates. Animals that received 0.15% LTS with Diet B showed resistance to such hepatic accumulation in all three oil pairs ($P<0.01$); and LTS with the polyunsaturated oils gave lower plasma cholesterol levels than LTS with the saturated oil ($P<0.01$). However, the different effects of unsaturated and saturated oils on the plasma-liver partitioning of cholesterol persisted with LTS feeding.

To observe whether an arylsulfonate has effects in the absence of dietary stress factors, the rat colony diet (Diet C) with no added cholesterol or glycocholate was fed to two groups of six weanling male rats, one of which received 0.15% LTS in the diet. After 15 wk, the control group had gained an average of 389 g while the group that received the LTS had gained an average of 398 g. All animals appeared to be normal. Liver weights in both groups were 2.7% of body weight. In another experiment, LTS and OTS, when fed in a low-cholesterol diet for 8 wk, produced no measurable lowering of cholesterol in plasma or liver. Thus, the arylsulfonate esters do not produce hypocholesterolemia when fed to normocholesterolemic rats.

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LETTER TO THE EDITOR

3-Hydroxy-3-Methylglutaric Acid and Orotic Acid Induced Fatty Liver in Rats

Sir: In our continuing efforts to establish the hypolipidemic activity of 3-hydroxy-3-methylglutaric acid (HMG), it was observed that HMG treatment of rats decreased serum lipids without increase in liver lipids (1,2). These observations were taken to rule out indirectly the possibility of HMG inhibiting the release of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) (2). The hypolipidemic activity of many drugs, including ethyl *p*-chlorophenoxy isobutyrate (CPIB), is mediated via a serum β-lipoprotein response, and they prevent orotic acid fatty liver (3,4). The object of the present study was to investigate if this was the case with HMG. CPIB was included in this study for comparative purposes. Twenty-four male albino rats on the average weighing about 160 g were divided into four groups. The basal group received the diet as described by Elwood et al. (3). In order to produce fatty liver conditions, the other three groups received the basal diet containing 1% orotic acid (E. Merck, Germany). One of these groups was treated i.p. with 50 mg HMG (Schwarz/Mann, Orangeburg, NY) per kg body weight in 1 ml saline and the other group received orally 0.3% CPIB (Ranbaxy, India) in the basal diet. The basal and control group received i.p. 1 ml saline only. The animals were sacrificed after 14 days treatment. Serum β-lipoprotein, VLDL + LDL, were separated by dextran sulfate precipitation method (5) and lipid estimated as reported earlier (2). Serum and liver lipids were extracted and estimated similarly.

In agreement with earlier reports that cholesterol and triglyceride levels of whole serum declined to a remarkable extent but liver cholesterol and triglyceride levels increased in animals receiving orotic acid (6), the decrease in lipids of serum β-lipoproteins was of the same magnitude as in whole serum. Essentially all the hypolipidemic drugs including CPIB preventing orotic acid fatty liver increased the intensity of serum β-lipoprotein bands on gel electrophoresis (3). Although not yet unequivocally established, this effect was related to their hypolipidemic action, and if it is so, the hypolipidemic effect of such drugs would seem to be mediated via serum β-lipoprotein. Simultaneous

TABLE I

Effect of 3-Hydroxy-3-Methylglutaric Acid (HMG) and Ethyl *p*-Chlorophenoxy Isobutyrate (CPIB) on Serum and Liver Lipids of Orotic Acid Fed Rats

Details	Basal group	Basal + orotic acid	Basal + orotic acid + 50 mg HMG/kg	Basal + orotic acid + 0.3% CPIB
Food consumption (g/day)	18.0 ±0.9[b]	17.3 ± 1.0	16.2 ± 2.0	18.0 ± 1.0
Weight gain/14 days	34 ± 2	88 ± 5	69 ± 5	29 ± 3
Liver weight (g)[a]	4.2 ± 0.1	8.5 ± 0.2	8.2 ± 0.2	4.9 ± 0.3
Whole serum lipids (mg/100 ml)				
Cholesterol	66 ± 3	33 ± 3	36 ± 2	53 ± 3[d]
Triglycerides	61 ± 5	34 ± 3	39 ± 5	47 ± 4[c]
Serum β-lipoprotein lipids (mg/100 ml)				
Cholesterol	45 ± 2	21 ± 1	22 ± 2	37 ± 2[c]
Triglycerides	49 ± 2	23 ± 3	28 ± 3	34 ± 3[e]
Liver lipids (mg/100 g)				
Cholesterol	250 ± 10	730 ± 60	630 ± 20	150 ± 10[c]
Triglycerides	1560 ± 70	4980 ± 22	4480 ± 130	1850 ± 70[c]

[a]The liver weight as percent of the final average body weight is 2.1%, 3.4%, 3.7%, and 2.7%, respectively, of basal group, basal + orotic acid + HMG, and basal + orotic acid + CPIB.

[b]Mean ± Standard Error expressed for six rats.

[c]Significantly different from basal + orotic acid fed group; $p < 0.0001$.

[d]$p < 0.001$.

[e]$p < 0.01$.

HMG administration had no significant effect in whole serum, serum β-lipoproteins and liver to overcome the lipid changes caused by orotic acid feeding. We confirm the earlier reports (3,4) that CPIB prevents the formation of orotic acid fatty liver in rats. A block in the secretion of hepatic triglycerides into the plasma resulting in the diminution of all classes of serum lipids has been suggested in orotic acid-induced fatty liver (7). Since HMG failed to prevent orotic acid fatty liver, the compound unlike CPIB is ineffective in overcoming the block in the secretion of hepatic triglycerides, and its hypolipidemic effect is at least not mediated through a β-lipoproteins response.

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Reduction of Blood and Liver Cholesterol in the Rat by Straight and Branched Chain Alkyl Amines

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ABSTRACT

The activities of a branched chain and several straight chain amines (C_{12} to C_{18} chain length), and the azasteroid 25-aza-5α-cholestane were compared with those of 20,25-diazacholesterol dihydrochloride, which is a potent hypocholesterolemic agent in the rat. These amines and azasteroids inhibit the Δ^{24}-sterol reductase system in the tobacco hornworm, *Manduca sexta* (L.), and also block the conversion of C_{28} and C_{29} plant sterols to cholesterol, with a resulting accumulation of desmosterol. The effects of these compounds in the rat were determined on body weight gain, cholesterol, desmosterol, and lipid composition of blood, feces, liver, and epididymal fat pad weight. The two azasteroids and the branched chain amine, N,N-dimethyl-3, 7,11-trimethyldodecanamine, had the greatest effect, reducing total plasma lipids and plasma sterols to approximately 40-50% of the levels in control rats and produced a concomitant increase in plasma and liver desmosterol. The branched chain dodecanamine caused a reduction in both feed consumption and body weight gain. The branched and straight chain dodecanamines also severely reduced epididymal fat pad weight. Our results demonstrate that the simple azasteroid, 25-aza-5α-cholestane, is a more potent inhibitor of cholesterol biosynthesis than the diazasterol and that the Δ^{24}-sterol reductase system in a mammal can be inhibited by simple, nonsteroidal, acyclic amines.

INTRODUCTION

Several new straight and branched chain secondary and tertiary amines were recently shown to have inhibitive effects upon the Δ^{24}-sterol reductase enzyme system involved in the conversion of plant sterols to cholesterol and to disrupt larval growth and development in the tobacco hornworm, *Manduca sexta* (L.) (1). These same effects were also observed in insects fed a number of different azasteroids (2,3), some of which are hypocholesterolemic in mammals (4) and birds (5). The possibility that these relatively simple alkyl amines, which are potent inhibitors of cholesterol production in insects, might also exert similar or related effects in mammals, prompted us to test these compounds in the rat. Accordingly, the effects of six straight and branched chain secondary and tertiary amines, containing a chain length of 12-18 carbons, and an azasteroid, 25-aza-5α-cholestane, were compared with those of 20,25-diazacholesterol dihydrochloride, a potent hypocholesterolemic agent in the rat (4).

EXPERIMENTAL PROCEDURES

The straight and branched chain amines were prepared as previously described (1), as was 25-aza-5α-cholestane (3); the 20,25-diazacholesterol dihydrochloride (SC-12937) was a gift of the G.D. Searle Company, Chicago, IL. The structures of the compounds and the concentrations are reported in Table I. All test compounds except the water soluble 20,25-diazacholesterol dihydrochloride were dissolved in acetone, mixed with the basal diet, and the solvent was removed by air drying. Groups of Sprague-Dawley rats (190-260 g) were fed ad libitum weighed quantities of ground rat chow, which contained 24.0% crude protein, 4.0% crude fat, and 4.5% crude fiber (Wayne Lab-Blox), and the appropriate concentration of test compound. Nine groups of twelve male rats were fed the amines at 1,000 ppm and the two azasteroids at 12.5 ppm of the diet for 3 weeks. Body weights were determined weekly and feed consumption was measured daily. Four rats from each of the nine groups were killed after 1, 2, and 3 weeks on the diets to determine the time course of changes produced by the amines. Voided feces of individual rats were collected during the 7 days before killing. Samples of blood, liver, and epididymal fat were taken for chemical analyses. Weighed samples of tissues were immediately homogenized in chloroform-methanol and extracted by the technique of Bligh and Dyer (6). The resulting lipid extract in chloroform was stored at -20 C after addition of butylated hydroxytoluene (0.1% of lipid weight) as recommended by Johnson (7). The

TABLE I

Amines and Azasteroids Tested for Effects on Cholesterol Biosynthesis in the Rat

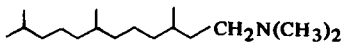
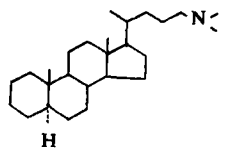
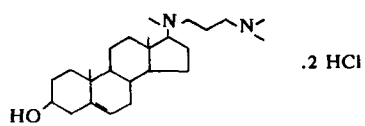
Dietary group	Test material	Structure	Concentration, ppm diet
1	N,N-Dimethyl-3,7,11-trimethyldodecanamine		1,000
2	N,N-Dimethyldodecanamine	$CH_3(CH_2)_{10}CH_2N(CH_3)_2$	1,000
3	N,N-Dimethyltetradecanamine	$CH_3(CH_2)_{12}CH_2N(CH_3)_2$	1,000
4	N,N-Dimethylhexadecanamine	$CH_3(CH_2)_{14}CH_2N(CH_3)_2$	1,000
5	N-Ethyloctadecanamine	$CH_3(CH_2)_{16}CH_2NH\ CH_2CH_3$	1,000
6	N,N-Dimethyloctadecanamine	$CH_3(CH_2)_{16}CH_2N(CH_3)_2$	1,000
7	25-Aza-5α-cholestane		12.5
8	20,25-Diazacholesterol dihydrochloride	 .2 HCl	12.5
9	Control		0

TABLE II

Effects of Amines and Azasteroids on Growth of Rats

Group	Initial body weight, g	Average 3-week gain, g	Change from basal ration, %	Slope of gain[a] g/week	95% Confidence interval
1	226	53	-62	16.51$^{D[a,b]}$	± 5.45
2	223	111	-21	36.98^{B}	± 4.61
3	212	117	-16	45.97^{B}	± 5.89
4	232	93	-34	30.82^{C}	± 6.38
5	230	74	-47	26.47^{C}	± 8.58
6	230	92	-34	32.66^{C}	± 8.86
7	234	116	-17	42.28^{B}	± 5.57
8	227	121	-14	41.08^{B}	± 4.90
9	230	140	0	47.92^{B}	± 4.45

[a]Heterogeneity of slopes by analysis of covariance with multiple comparison among slopes by Student-Newman-Keuls test.

[b]Values bearing different superscripts differ significantly ($p<0.05$).

TABLE III

Effects of Amines and Azasteroids on Feed Consumption and Efficiency of Gain of Rats

Group	Average 3-week feed consumption, g	Change from basal ration, %	Efficiency of gain[a], %	As % of control gain
1	394$^{B[b]}$	-26	13.5^{C}	51.5
2	474^{C,E}	-11	23.4^{B}	89.3
3	470^{C,E}	-12	24.9^{B}	95.0
4	457^{C}	-14	20.4^{B,C}	77.9
5	403^{B}	-25	18.4^{B,C}	70.2
6	426^{B}	-20	21.6^{B,C}	82.4
7	498^{D,E}	-7	23.3^{B}	88.9
8	508^{D,E}	-5	23.8^{B}	90.8
9	534^{D}	0	26.2^{B}	100.0

[a]Efficiency of gain = 100 x (average 3-week body weight gain in g)/(average 3-week feed consumption in g).

[b]Values bearing different superscripts differ significantly ($p<0.05$).

fat content of tissues and diets was determined gravimetrically after ether extraction by using the Goldfisch extraction apparatus.

Plasma and liver sterols were examined by gas liquid chromatography (GLC) after extraction and purification as previously described (8). The GLC analyses were made on a glass column containing 1% OV-17 coated on Gas-Chrom P on a Barber-Colman model 10 gas chromatograph using cholestane as an internal standard. Free and total cholesterol in liver were determined after digitonin precipitation of the cholesterol (9). Total plasma lipids were determined by a colorimetric method based on the sulfophosphovanillin reaction (10,11). Lipids for detailed fatty acid analysis were extracted from feces with ethyl ether using the Goldfisch fat extraction apparatus. Methyl esters were prepared with methanolic-HCl-dimethoxypropane (12) and the fatty acid composition was determined by programmed GLC with a Model 7620 Hewlett Packard gas chromatograph, using a 0.6 cm x 183 cm glass column packed with 10% EGSS-X on Gas-Chrom P (100/120 mesh).

Statistical comparisons were made by using either the Students *t*-test with correction for unequal group size or by analysis of variance with mean separation by Student-Newman-Keuls test.

RESULTS

Weight Gains and Efficiency of Food Utilization

The comparative growth of rats fed the amines and azasteroids is shown in Table II. The two azasteroids had no apparent effect on rat growth at 12.5 ppm concentration in the diet (groups 7 and 8). The average 3-week gain in body weight was severely reduced by the branched chain tertiary dodecanamine (group 1), the C_{16} and C_{18} straight chain tertiary amines (groups 4 and 6), and the C_{18} straight chain secondary amines (group 5) when each was fed at 1,000 ppm. Reduced feed consumption paralleled the growth response (Table III);

TABLE IV

Effects of Amines and Azasteroids Upon Organ Weights of Male Rats[a]

Group	Body weight g	Liver		Testes		Epididymal fat pads	
		Weight g	% Body weight g/100 g	Weight g	% Body weight g/100 g	Weight g	% Body weight g/100 g
1	282	$11.67^{A,C}$	$4.1^{A,B}$	2.88^{A}	1.02^{A}	$1.62^{B,C}$	0.57^{A}
2	338	$12.85^{A,C}$	$3.8^{A,B}$	2.83^{A}	$0.84^{A,C}$	$1.36^{B,C}$	0.52^{A}
3	352	$14.04^{A,C}$	$4.0^{A,B}$	3.02^{A}	$0.86^{A,C}$	$2.54^{A,C}$	0.72^{A}
4	316	$11.64^{A,C}$	$3.7^{A,B}$	2.95^{A}	$0.89^{A,C}$	$1.96^{B,C}$	0.63^{A}
5	308	$11.04^{B,C}$	$3.6^{B,C}$	2.92^{A}	0.95^{A}	$1.92^{B,C}$	0.62^{A}
6	330	$12.60^{A,C}$	$3.8^{A,B}$	2.77^{A}	$0.85^{A,C}$	$2.28^{A,C}$	0.70^{A}
7	359	$13.65^{A,C}$	$3.8^{A,B}$	2.60^{A}	$0.69^{B,C}$	$2.48^{A,C}$	0.69^{A}
8	347	14.70^{A}	4.2^{A}	3.02^{A}	$0.87^{A,C}$	$2.18^{A,C}$	0.63^{A}
9	376	$13.66^{A,C}$	$3.6^{A,B}$	3.16^{A}	$0.84^{A,C}$	3.27^{A}	0.87^{A}

[a]Values bearing different superscripts differ significantly ($p<0.05$).

TABLE V

Fatty Acid Composition of Rat Feces

Group	Total lipid (% dry mass)	Fatty acid (weight %)						
		<16:0[a]	16:0	18:0	18:1	18:2	18:3	Others[b]
1	$3.19^{C,D}$[c]	6.7	19.7^{C}	14.0^{D}	32.3^{D}	15.2^{C}	$4.0^{C,D}$	8.1
2	2.64^{D}	6.6	18.6^{C}	10.3^{D}	34.4^{D}	16.4^{C}	3.4^{D}	10.3
3	2.86^{D}	6.7	17.9^{C}	9.8^{D}	35.2^{D}	17.4^{C}	$3.6^{C,D}$	9.4
4	2.95^{D}	7.1	17.2^{C}	11.6^{D}	31.4^{D}	18.6^{C}	$4.6^{C,D}$	9.4
5	3.71^{C}	6.2	18.6^{C}	19.7^{C}	26.7^{C}	13.2^{C}	$4.7^{C,D}$	9.0
6	3.37^{C}	9.0	17.2^{C}	13.0^{D}	32.8^{D}	16.0^{C}	$3.5^{C,D}$	8.3
7	3.70^{C}	6.1	20.4^{C}	12.0^{D}	30.9^{D}	16.7^{C}	$4.4^{C,D}$	9.5
8	3.30^{C}	5.8	17.9^{C}	7.7^{D}	37.2^{D}	14.8^{C}	6.1^{C}	10.5
9	2.83^{D}	5.8	18.2^{C}	8.7^{D}	35.6^{D}	15.7^{C}	$4.8^{C,D}$	11.4

[a]Includes 14:0, 14:0 branched (br), 14:1, 15:0, 16:0 br, 16:1.

[b]Includes 17:0, 18:0 br, 20:0, and longer chain fatty acids.

[c]Values bearing different superscripts differ significantly ($p<0.05$).

TABLE VI

Concentration of Plasma Total Lipids After Feeding Rats Amines and Azasteroids for 3 Weeks

Group	Total lipids[a]	
	mg/100 ml	% of Control
1	$157^{B,C}$	60
2	$192^{B,C}$	73
3	256^{A}	97
4	225^{A}	86
5	$204^{A,C}$	78
6	212^{A}	81
7	$138^{B,C}$	52
8	$170^{B,C}$	65
9	263^{A}	100

[a]Values bearing different superscripts differ significantly ($p<0.05$).

when the efficiency of the body weight gain was examined (body weight gain: feed intake), the branched chain tertiary dodecanamine was seen to cause the most severe reduction of growth. Rats fed the branched dodecanamine were only about 50% as efficient as controls in converting feed energy to body tissue.

Effects of Amines and Azasteroids Upon Organ Weights

After 3 weeks, the rats fed the amines that caused growth reduction had smaller livers and testes than the controls (Table IV) but, statistically, they were not significantly different from controls. The amines exhibited their greatest depressive effects upon epididymal fat pad weights (Table IV). In fact, all test compounds depressed epididymal fat pad weights, suggesting that lipid metabolism was severely affected. When expressed on a per unit of body weight basis, however, the epididymal fat pad weights of treated rats were not significantly different from those of control rats.

Changes in Fatty Acid Composition of Feces

After 3 weeks, the amount of fat (Table V) increased in the feces of 2 of the 6 groups fed the amines (groups 5, 6) and in the feces of the

TABLE VII

Effects of Amines and Azasteroids on Plasma Sterols in the Rat[a]

Group	Total sterol (mg/100 ml)			% Desmosterol		
	1 Week	2 Weeks	3 Weeks	1 Week	2 Weeks	3 Weeks
1	9.8^{C}	10.4^{D}	$22.8^{B,C}$	20.9^{C}	17.0^{C}	22.2^{C}
2	20.6^{B}	$35.3^{A,B}$	$41.8^{A,C}$	- D	$<1^{D}$	$<1^{D}$
3	$16.9^{B,C}$	$40.7^{A,B}$	$29.8^{A,C}$	3.6^{D}	4.1^{D}	5.4^{D}
4	35.6^{A}	$31.2^{B,C}$	$25.4^{B,C}$	3.0^{D}	4.1^{D}	1.8^{D}
5	$24.5^{B,D}$	$42.9^{A,B}$	45.5^{A}	0.7^{D}	$<1^{D}$	2.2^{D}
6	$30.7^{A,D}$	46.1^{A}	$31.2^{A,C}$	1.3^{D}	$<1^{D}$	1.8^{D}
7	11.4^{C}	12.3^{D}	$25.0^{B,C}$	43.4^{A}	65.9^{A}	73.7^{A}
8	$17.1^{B,C}$	22.8^{C}	$27.6^{A,C}$	32.8^{B}	42.1^{B}	44.9^{B}
9	$32.1^{A,D}$	$38.3^{A,B}$	45.6^{A}	- D	- D	- D

[a]Values bearing different superscripts differ significantly ($p<0.05$). (-) Indicates desmosterol did not occur at a detectable level under our gas liquid chromatographic conditions.

TABLE VIII

Effects of Amines and Azasteroids on Liver Sterols in the Rat[a]

Group	Total sterol (mg/g)			% Desmosterol		
	1 Week	2 Weeks	3 Weeks	1 Week	2 Weeks	3 Weeks
1	$1.82^{A,B}$	1.63^{B}	1.99^{A}	32.5^{B}	30.8^{C}	22.6^{C}
2	2.05^{A}	$2.26^{A,B}$	2.27^{A}	1.1^{C}	7.6^{D}	2.9^{D}
3	$1.77^{A,B}$	2.36^{A}	2.10^{A}	7.3^{C}	8.5^{D}	6.3^{D}
4	2.26^{A}	$2.13^{A,B}$	2.25^{A}	3.8^{C}	$3.7^{D,E}$	4.9^{D}
5	2.13^{A}	2.39^{A}	1.59^{A}	- C	- E	4.3^{D}
6	1.98^{A}	$2.18^{A,B}$	2.01^{A}	- C	1.2^{E}	2.1^{D}
7	$1.77^{A,B}$	1.77^{B}	1.54^{A}	57.4^{A}	72.4^{A}	77.8^{A}
8	1.42^{B}	$1.88^{A,B}$	1.56^{A}	39.8^{B}	39.9^{B}	46.7^{B}
9	1.98^{A}	$2.13^{A,B}$	2.20^{A}	- C	- E	- D

[a]Values bearing different superscripts differ significantly ($p<0.05$). (-) Indicates desmosterol did not occur at a detectable level under our gas liquid chromatographic conditions.

rats fed the azasteroids (groups 7, 8). However, the fatty acid composition of the excreted lipid did not change greatly, and the only significant quantitative change appeared to be in the stearic acid (18:0) content. There was a trend towards a greater proportion of 18:0 in the fat excreted by rats of groups 1, 5, 6, and 7 and a slightly lower proportion of longer chain fatty acids but this change in 18:0 was statistically different only for group 5.

Plasma Total Lipids

Table VI shows that almost all of the amines and azasteroids had a depressive effect upon plasma total lipid content. The rats fed the branched chain dodecanamine (group 1), the C_{12} tertiary-amine (group 2), azacholestane (group 7), and diazacholesterol (group 8), exhibited progressive declines in plasma lipid values. After ingesting the compounds for 3 weeks, plasma lipid concentrations had decreased by 35% to 48%.

Plasma and Liver Sterols

The compounds that were most effective in lowering plasma lipids (groups 1, 7, 8) were also most effective in lowering total plasma sterols (Table VII). This decrease was apparent after 1 week of feeding, and in all 3 groups plasma sterols were reduced to one-half the control level. Plasma cholesterol was reduced and the plasma desmosterol increased concomitantly to 22%, 74%, and 45% of the total sterol by the branched chain dodecanamine, azacholestane, and diazacholesterol, respectively (Table VII). The azacholestane caused a progressive accumulation of desmosterol to 74% of the total sterols, which was in marked contrast to the other two potent inhibitors of cholesterol synthesis (groups 1 and 8), which exerted near-maximal effects 1 week after administration. The other amines caused smaller increases in the desmosterol levels in the plasma from trace amounts to 1-5% of total sterols.

Liver sterols showed a pattern quite similar to that of the plasma sterols (Table VIII). The branched dodecanamine (group 1), azacholestane (group 7), and diazacholesterol (group 8) were most effective in lowering total sterol

levels and in increasing the proportion of liver desmosterol. Quantitatively, the amount of inhibition of cholesterol synthesis was the same as in the plasma, and desmosterol was increased to 23%, 78%, and 47% of total sterols by the branched dodecanamine, azacholestane, and diazacholesterol, respectively. As determined by digitonin precipitation, all of the liver cholesterol and desmosterol appeared to be nonesterified.

DISCUSSION

Azacholestane, an azasteroid that inhibits the Δ^{24}-sterol reductase in insects (3), was demonstrated to be a potent inhibitor of the Δ^{24}-sterol reductase system in a mammal, the laboratory rat. In addition, a branched chain dodecanamine and several straight chain secondary and tertiary amines, all of which exert inhibitive effects upon development and metamorphosis and the Δ^{24}-sterol reductase system in tobacco hornworm larvae (1), were also found to inhibit cholesterol biosynthesis in the rat with a resultant accumulation of desmosterol in plasma and liver. As far as we know, this is the first report of acyclic compounds having such activity in a mammal. It is also significant that no mortality was observed with any of these compounds at the dosage levels tested.

Several of these simple amines that lack the steroid nucleus exerted depressive effects upon growth of the rats, upon the efficiency of feed utilization, and upon the size of the epididymal fat pad stores. The epididymal fat pad is a sensitive indicator of adipose tissue metabolism and readily reflects lipogenesis and lipolysis in the body (13). It is possible that the decrease in weight of the eipididymal fat pad could merely represent a compensatory mechanism to mobilize caloric resources in those rats in which growth was severely reduced, rather than an interference with lipid metabolism. However, the fat pads also decreased greatly in several groups in which body weight gains were not different from those of the controls. We also noted a decrease in total plasma lipids and an increase in excretion of lipid in the feces. All these effects, in addition to the inhibitive effects on cholesterol biosynthesis, suggest that a more general interference with lipid metabolism was induced by the amines. Further information concerning this effect might be obtained more directly by determination of the activity on the specific enzyme systems involved in lipid metabolism.

The effects of the amines and azasteroids differed: 25-azacholestane and 20,25-diazacholesterol at 12.5 ppm in the diet did not cause weight losses although they induced a large decrease in epididymal fat pad weight and in cholesterol biosynthesis; the branched chain dodecanamine at 1,000 ppm caused the greatest depression in growth and was more effective in interfering with cholesterol synthesis than the other acyclic compounds. Interestingly, the 25-azacholestane was substantially more potent than the diazacholesterol in these tests.

The decrease in plasma and liver cholesterol and the concomitant accumulation of desmosterol in the rat indicate a block in the Δ^{24}-sterol reductase system. The potent activity exhibited by the branched chain dodecanamine indicates that extremely simple, nonsteroidal compounds can successfully block the mammalian cholesterol biosynthetic pathway, and this is the first demonstration in a mammal of interference with cholesterol biosynthesis by structurally simplified nitrogen-containing compounds that contain no ring substitutents. In related studies, we recently reported inhibition of the Δ^{24}-sterol reductase system by these same compounds in an avian species, the chicken (14). In rhe chicken, the C_{12} branched chain amine, 25-azacholestane, and 20,25-diazacholesterol were found to be effective in causing reduction of plasma and egg cholesterol and concomitant increases in desmosterol.

These comparative studies then have demonstrated that an extremely simple steroid, 25-azacholestane, and a simple branched chain amine, N,N-dimethyl-3,7,11-trimethyldodecanamine, are potent inhibitors of Δ^{24}-sterol reductase in several species. The reduction in cholesterol and accumulation of demosterol suggest interference with a similar enzyme system in insects (1,3), birds (14), and mammals. The information obtained with these structurally different classes of tertiary amines may well serve to guide the development of new classes of vertebrate hypocholesterolemic and/or hypolipemic agents.

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Lipid Synthesis in Cultured Human Embryonic Fibroblasts

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ABSTRACT

We describe here the pathways by which human embryonic fibroblasts synthesize lipids. In these studies, we quantitated the phospholipids by their phosphorus content and by their acyl components. These determinations defined both the chemical composition of the cellular membranes as well as their metabolic turnover. Using radiolabeled precursors, we have shown (a) synthesis of the glycerol moiety via glycolysis and the action of glycerokinase, (b) utilization of both exogenously added and endogenously synthesized fatty acids, (c) synthesis de novo of phosphatidyl choline and phosphatidyl ethanolamine from their base precursors, and (d) the methylation of phosphatidyl ethanolamine yielding phosphatidyl choline. Dividing cells synthesized phosphoglyceride more rapidly than cells in the stationary phase. However, considerable turnover of cellular lipid did occur in the stationary phase.

INTRODUCTION

During herpesvirus multiplication in host cells, the virus acquires its envelope by budding from the inner nuclear membrane. Ben-Porat and Kaplan reported that the virus envelope was derived from a site of newly-formed lipid in the nuclear membrane (1). Since it is generally accepted that the endoplasmic reticulum is the major, if not exclusive, site for phosphoglyceride synthesis (with the exception of cardiolipin) (2), it would seem reasonable that herpes simplex virion (HSV) stimulates the transfer of phosphoglyceride from the endoplasmic reticulum to the nuclear membrane. This transfer could be similar to that described by Wirtz and Zilversmit (3) or may involve an unknown mechanism. Data reported by Ben-Porat and Kaplan (1) also showed a decrease in the total amount of labeled lipid in the cytoplasmic fraction after virus infection of host cells. It is not possible from their study to determine whether there had been a net loss of preformed phosphoglyceride from the membrance or if new cytoplasmic membrane synthesis from nonlabeled precursors was inhibited after infection. Asher et al. (4) did not find stimulation of [^{3}H] choline incorporation into phosphoglycerides of cultured monkey kidney cells after HSV infection. From these data, it is reasonable to assume there would be a net loss in the lipids of the endoplasmic reticulum during virus multiplication.

To elucidate the metabolic events involved in herpesvirus-host cell interaction, it is imperative to determine the existence of key metabolic pathways of lipid synthesis and their interconversion in host cells. Therefore, experiments were designed to investigate in hyman embryonic fibroblasts (HEF) (a) the origin of the glycerol phosphate from both glycolysis and by the action of glycerokinase, (b) the synthesis of fatty acids from acetate and glucose, (c) the incorporation of added fatty acids (both saturated and unsaturated) and endogenously synthesized fatty acid, (d) the synthesis de novo of phosphatidyl choline (PC) and sphingomyelin (Sph) from choline and phosphatidyl ethanolamine (PE) from ethanolamine, and (e) the methylation of PE to form PC. Also, we contrasted the incorporation of labeled ethanolamine into total lipids in growing phase and stationary phase cells.

TABLE I

Protein and Phospholipid Content of HEF

	Hour					
	0	4	8	24	28	32
Number of cells x 10^{-5}	6.3	7.2	7.4	8.3	11.0	12.0
μg Protein	400	400	436	404	516	520
nmoles Phospholipid	24.1	24.5	33.2	31.7	50.8	47.5
μg Protein/10^5 cells	63	56	59	49	47	43
nmoles Phospholipid/10^5 cells	3.8	3.4	4.5	3.8	4.6	3.9

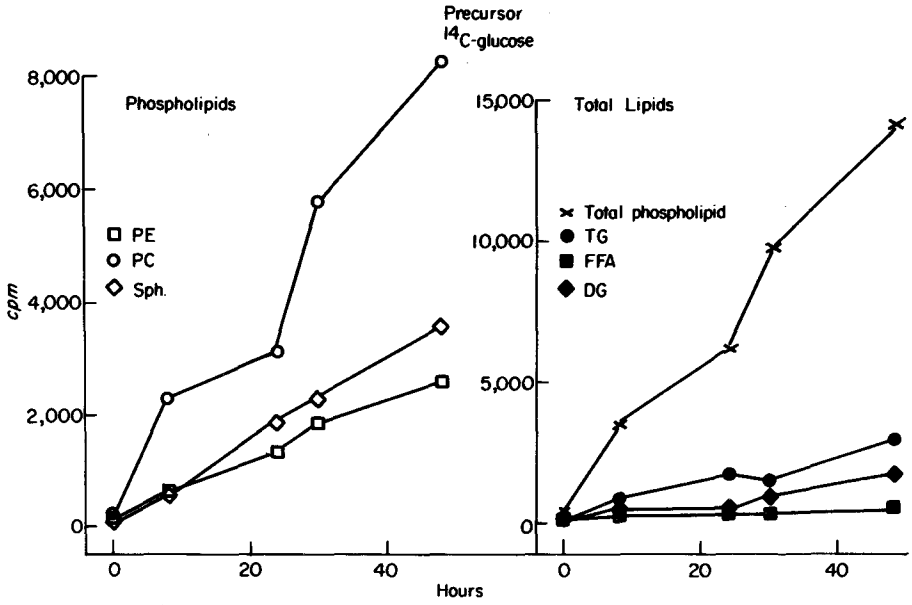

FIG. 1. Analysis of continuous uptake of [^{14}C] glucose (1 μCi/ml); Sp. Act. = 190 mCi/mmole) into phospholipids and total lipids extracted from stationary phase (100% sheeted monolayers) HEF cell monolayers. The cultures were overlaid with 2 ml of growth medium supplemented with [^{14}C] glucose. At various times after incubation at 37 C, some of the cultures were harvested by scraping the cells into the overlay medium and washing each culture dish with 0.5 ml of 0.01 M phosphate buffered saline pH 7.0 (PBS). The wash fluids were pooled with the cell suspension. The cells were pelleted (450 x g, 5 min), and suspended in 1 ml PBS. The cell lipids were extracted and analyzed as described in Experimental Procedures.

EXPERIMENTAL PROCEDURES

Cell Cultures and Media

Secondary human embryonic fibroblast cell cultures purchased from Flow Laboratories, Rockville, MD, were used between passages 5 and 17 and propagated as previously described (5). Briefly, the cell growth medium consisted of Eagle's minimal essential medium with Hank's base salts (Grand Island Biological Company, Grand Island, NY) supplemented with 10% heat-inactivated (56 C, 30 min) fetal calf serum (FCS), 10% tryptose phosphate broth, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.075% $NaHCO_3$, and 2 mM L-glutamine. Cells were serially passaged in 75 cm^2 plastic flasks (BioQuest, Oxnard, CA). For experiments, about 1.0 x 10^5 cells were suspended in 2 ml of growth medium with Earle's base salts and subcultured to each plastic 35 cm petri dish (Flow Laboratories, Rockville, MD). For lipid quantitation, usually about 1.0 x 10^7 cells were grown in culture bottles. All cell cultures were incubated at 37 C in a humidified incubator with a 5% CO_2 atmosphere. Experiments in which radiolabeled fatty acids were incorporated, the $CHCl_3$ solution containing the fatty acid was dried, the media added, and the fatty acid suspended by ultrasonic irradiation. The protein content of the cells was quantitated by the procedure of Lowry et al. (6) using bovine serum albumin as the standard.

Source of Radiolabeled Compounds

1-[^{14}C]-acetate, methyl-[^{14}C]-methionine, and 1,2-[^{14}C]-choline were purchased from New England Nuclear, Boston, MA. 9,10-[^{3}H]-palmitate, 1-[^{14}C]-linoleate, 1-[^{3}H]- and 1,2-[^{14}C]-ethanolamine were purchased from Amersham/Searle, Arlington Heights, IL. ICN supplied the uniformly labeled glucose and glycerol.

Extraction and Separation of the Lipids.

The lipids of the cells were extracted by the method of Bligh and Dyer (7). Aliquots of the $CHCl_3$ extract were counted to determine the total precursor incorporation into lipid and chromatographed on Silica Gel H plates in the chloroform-methanol-acetic acid-water system (60:40:5:4, v/v) or the chloroform-methanol-7N ammonia (60:40:5, v/v) to separate the phosphoglycerides. The neutral lipids were separated in the ether-ligroin (bp 63 C-75 C) formic acid (70:30:1.5, v/v) system. The lipids were visualized by staining with I_2 vapor. The

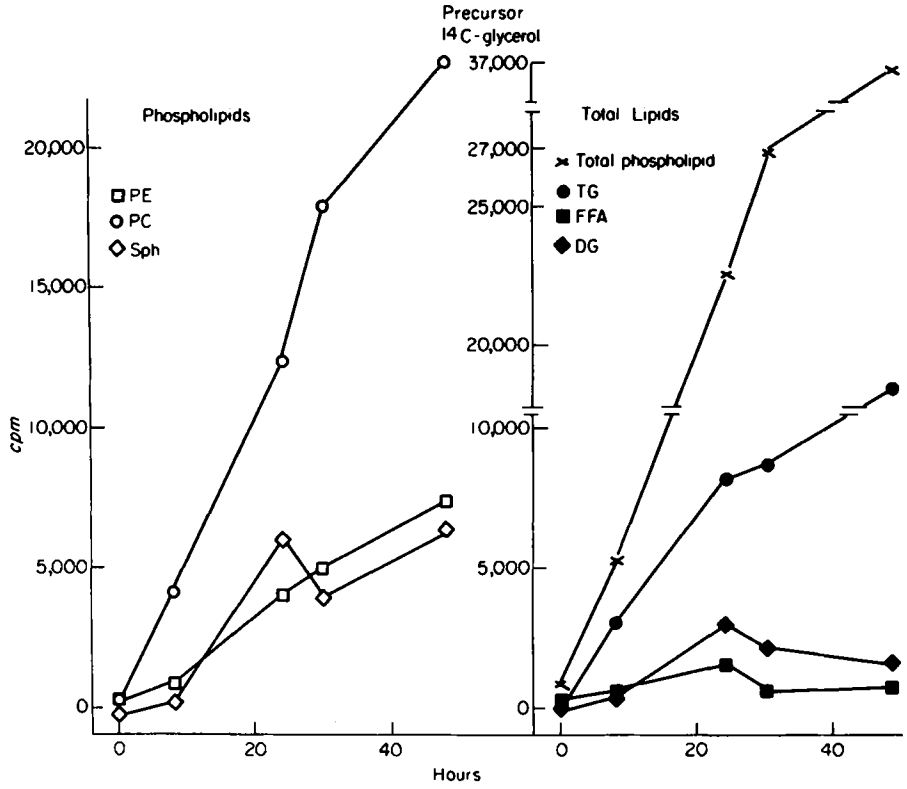

FIG. 2. Analysis of continuous uptake of [^{14}C] glycerol (1 μCi/ml; Sp. Act. = 16 mCi/mmole) into phospholipids and total lipids extracted from stationary phase (100% sheeted monolayers) HEF cells. See legend under Figure 1 for Experimental Procedures.

silica gel which contained the lipid was scraped into a scintillation vial and counted using a toluene-Triton x 100-H_2O (2:0.2:1, v/v) scintillation cocktail. The lipids were eluted from the silica gel by a mixture of $CHCl_3$-methanol-H_2O (2:1:0.3, v/v) for phosphorus analysis (8). Those samples taken for analysis by gas liquid chromatography were treated in the same way except that they were detected by rhodamine spray rather than by I_2 vapor. The methylation and gas chromatography are described elsewhere (9). An internal standard of behenic acid was used for quantitation.

To measure glucose incorporation into the fatty acids of the total glycerides, the $CHCl_3$ cell extract was dried and redissolved in 1.0 ml of 0.1 M NaOH in methanol. The sample was incubated for 1 hr at 37 C, 1 ml of 0.2 M HCl was added and the fatty acid was extracted in pentane. Aliquots of each phase were counted by scintillation spectrometry to measure the amount of label in the glycerol moiety (water-methanol phase) and acyl moiety (pentane phase). Chromatography of the initial $CHCl_3$ extract indicated that less than 3% of the label was free fatty acid.

RESULTS AND DISCUSSION

We first determined the relationship between the number of cells in culture and their protein and phospholipid content. Table I shows that over a 32 hr period of culture there was a doubling in the number of cells. Since the ratio of protein and phosphoand phospholipid content to cell number remains constant, we concluded that the cellular membranes are not changing appreciably during culture. This is also found when the composition of the membrane lipids is analyzed. In the experiments reported here, there was some variability in the number of cells used; therefore, the results presented are from a representative experiment rather than an average of all. With the exception of the cell counting, all determinations were done in at least three experiments.

Both uniformly labeled [^{14}C] glucose and [^{14}C] glycerol were incorporated into the cellular lipids of growing phase HEF (Fig. 1 and 2). The major class of products was phospholipid and the major phospholipid was PC. Considerable amounts of labeled precursor appeared in Sph. Since this phospholipid does not

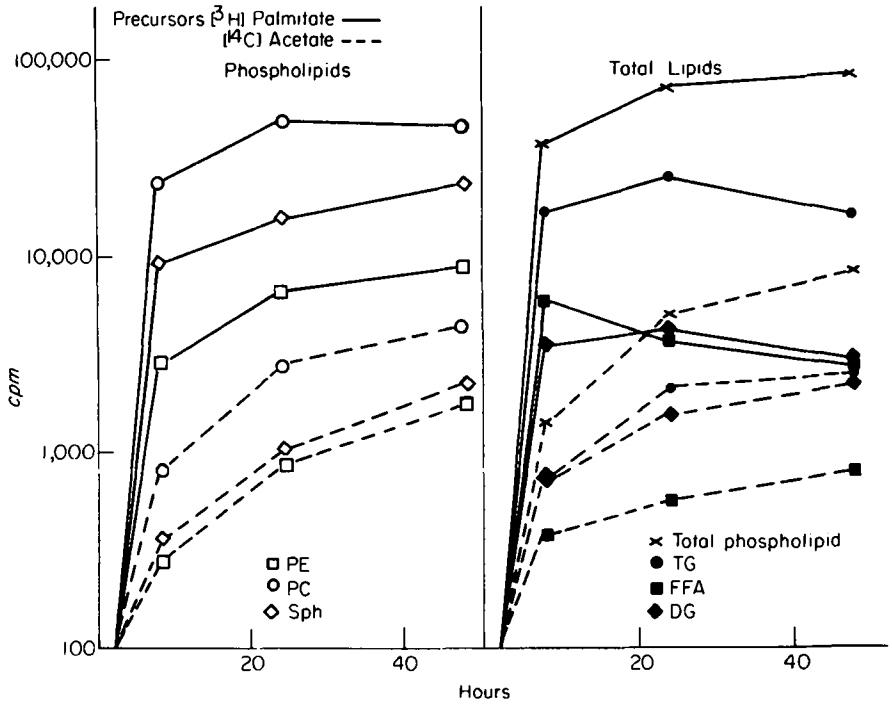

FIG. 3. Analysis of continuous uptake of [^{3}H] palmitate (5 μCi/ml; Sp. Act. = 477 mCi/mmole) and [^{14}C] sodium acetate (1 μCi/ml; Sp. Act. = 60 mCi/mmole) into phospholipids extracted from stationary phase (100% sheeted monolayers) HEF cells and total lipids. See legend under Figure 1 for Experimental Procedures.

contain glycerol, the labeled precursor would most likely be the acyl chain or the sphingosine derived from palmitic acid. Saponification of the total lipids demonstrated that 20% of the labeled precursor was in the acyl groups of the lipids and 80% in water soluble products, presumably glycerol. Small amounts of free fatty acid accumulated with either precursor which suggests that once synthesized, they are rapidly incorporated into lipids (cf. Figs. 3 and 4). An appreciable amount of triglyceride (TG) was also synthesized; relatively more labeled glycerol was incorporated into TG than labeled glucose (about 30% vs. 15%). These data suggest that a pool of diglyceride (DG) arising from glycolysis was used primarily for phospholipid synthesis whereas DG drived from the action of glycerol kinase was utilized to somewhat a greater extent for neutral lipid synthesis. Since the amounts of glycerol and glucose present in the media differed so greatly, no attempt was made to quantitate the relative incorporation of the two.

To demonstrate that the cells could utilize both exogenous as well as synthesized fatty acids, we contrasted the incorporation of [^{3}H] palmitate and [^{14}C] acetate (Fig. 3). Results showed a very rapid incorporation of [^{3}H] palmitate between 0 and 10 hr; after 24 hr

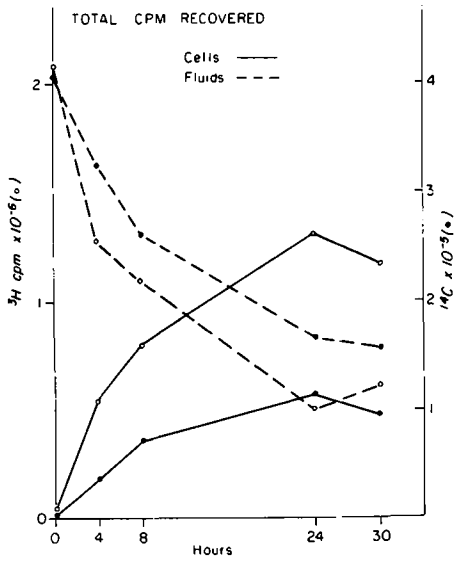

FIG. 4. Analysis of continuous uptake of [^{3}H] palmitate (10 μCi/ml; Sp. Act. = 477 mCi/mmole) and [^{14}C] linoleate (2 μCi/ml; Sp Act. = 61 mCi/mmole) into phospholipids and total lipids extracted from HEF cells and supernatant fluids (initial 1.1 x 10^7 cells). These data are of aliquots of each sample counted directly. The designations are (○), [^{14}C]; (O), [^{3}H].

there was only a slight increase in palmitate incorporation. At 8 hr, little free [^{3}H] palmitate was recovered in the cells; most had been incorporated into the lipids. On the other hand, [^{14}C] acetate incorporation into lipid continued to increase between 0 and 48 hr. We did not determine whether acetate was incorporated by synthesis de novo or by acyl elongation (10). Similar to the findings with [^{14}C] glycerol and [^{14}C] glucose, PC was the major lipid labeled. More labeled precursor was found in Sph than in PE which indicates that the choline containing lipids are very active metabolically. Considerable label was found in TG as well, although there was a decrease in [^{3}H] TG and [^{3}H] DG after 24 hr. The total recovery of [^{3}H] lipid from the cells and the growth medium decreased from 4.6 x 10^5 cpm to 3.0 x 10^5 cpm during the observation period which possibly could be the result of fatty acid oxidation. Since there was a net increase in the total amount of [^{3}H] phospholipid, it is possible that some [^{3}H] TG and [^{3}H] DG serves as precursor for [^{3}H] phospholipid.

The next experiment was designed to compare the kinetics of incorporation of a saturated ([^{3}H] palmitate) with an unsaturated acid ([^{14}C] linoleate). This comparison required that the specific activities of the precursor fatty acids and product phospholipids be determined. The cells and supernatant fluids were separated and the free fatty acids and phospholipids isolated. Aliquots of the purified compounds were counted in a scintillation counter and the methyl esters of the acyl groups formed. Table II shows the acyl group composition of the isolated lipids as the average of five time periods of incubation. The only changes that occurred during growth are noted by an asterisk. A striking feature of these results is the lack of polyunsaturated fatty acid normally found in mammalian cells. The major changes were an increase in the percentage of 18:2 in phosphatidyl serine + phosphatidyl inositol (PS + PI) from 14.4 to 24.9, a decrease in cellular free fatty acid from 22.2 to 10.7, and a decrease in the 18:1 of both pools of free fatty acid from about 24 to about 15. The changes that are concomitant with these did not occur in a single acid and, therefore, did not cause a change of more than a few percent.

The [^{3}H] palmitate entered the cell more rapidly than did the [^{14}C] linoleate (Fig. 4). When the data of this figure is combined with that of Table I, this means that 4-5 times more palmitate was taken up than 18:2 on a mass basis. Further, there was a greater recovery of [^{3}H] than [^{14}C] as lipid. (The loss presumably

TABLE II

Acyl Composition of HEF Phosphoglycerides and Free Fatty Acids

	Fatty acid composition[a]										
	14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:4	22:4	22:5	22:6	X
Cells												
Phosphatidyl choline	4.5	23.6	7.3	18.8	25.7	8.6	.2	3.9	tr[b]	2.0	1.0	3.7
Phosphatidyl ethanolamine	8.1	18.4	9.4	18.9	19.4	7.5	tr	6.8	3.5	3.7	4.1	4.2
Phosphatidyl serine + Phosphatidyl inositol	5.7	19.1	8.4	22.2	15.8	17.7[c]	tr	2.5	tr	2.0	2.9	7.8
Fatty acid	7.2	23.8	10.0	17.5	18.7[c]	11.1[c]	.8	tr	tr	1.4	1.5	5.7
Fluids												
Fatty acid	8.4	19.4	8.5	14.4	22.2[c]	11.2	2.2	2.2[c]	ND[d]	2.1	2.5	6.4

[a]Presented as weight percentages.

[b]tr = trace.

[c]These areas change significantly during cell growth.

[d]ND = none detected.

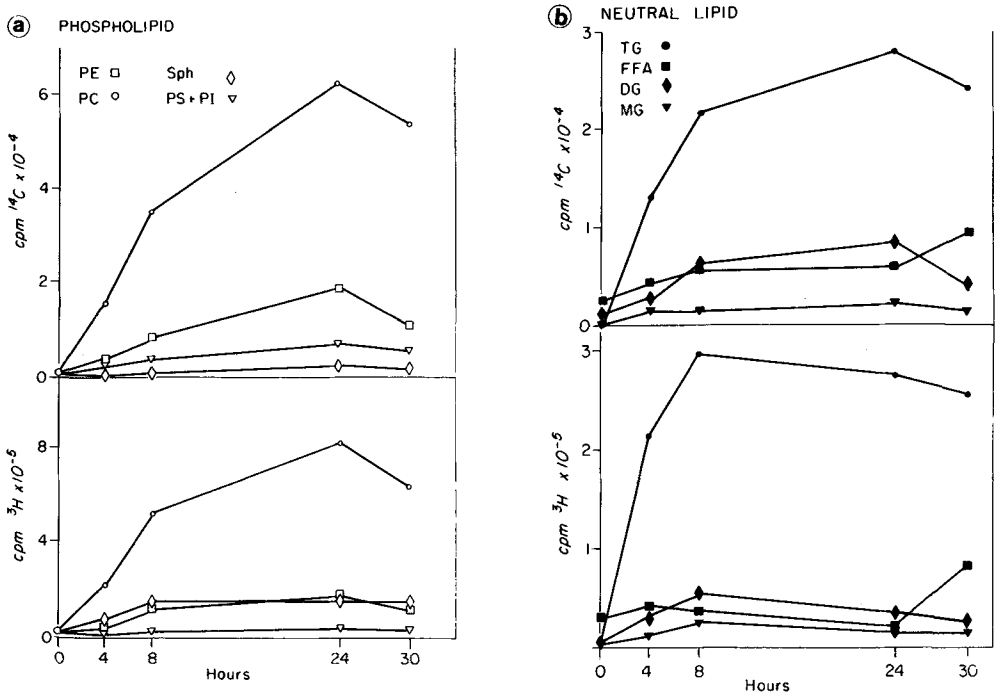

FIG. 5. The radioactivity of the phospholipids (panel a) and neutral glycerides (panel b) separated by thin layer chromatography. These samples were from the experiment described in Figure 4. Aliquots (0.1 ml) of each lipid extracted (about 3 ml) were counted.

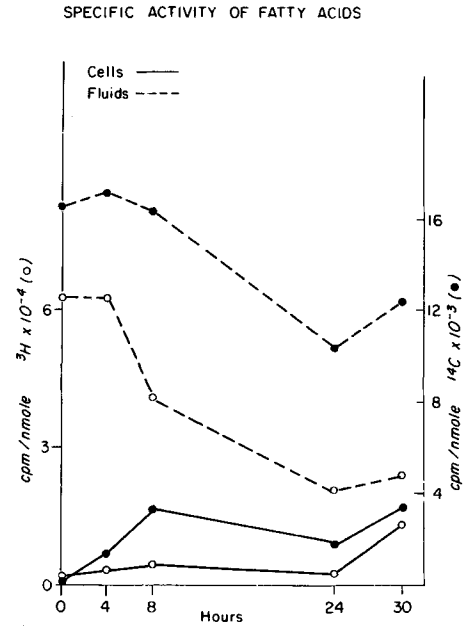

FIG. 6. The free fatty acids isolated in the experiment in Figure 5 were quantitated by gas liquid chromatography. The amount of palmitic acid was divided into the total [^{3}H] counts while the amount of linoleic acid was divided into the [^{14}C] counts.

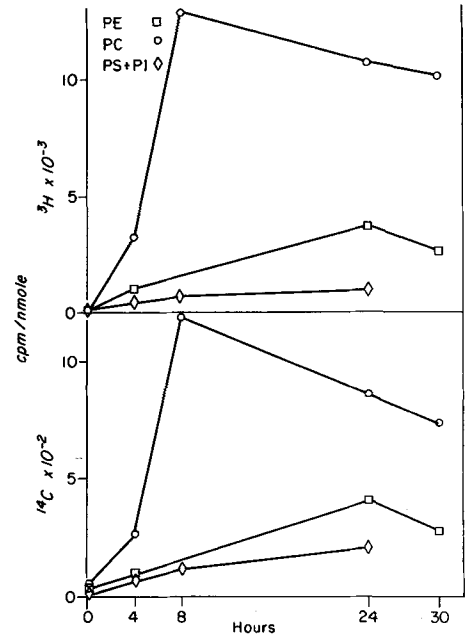

FIG. 7. The total amount of each phosphoglyceride, quantitated by gas liquid chromatography, was divided by the [^{3}H] counts (top) and [^{14}C] counts (bottom). The data were calculated on the basis of the individual fatty acids as well. Those results are not presented but are discussed in the Results.

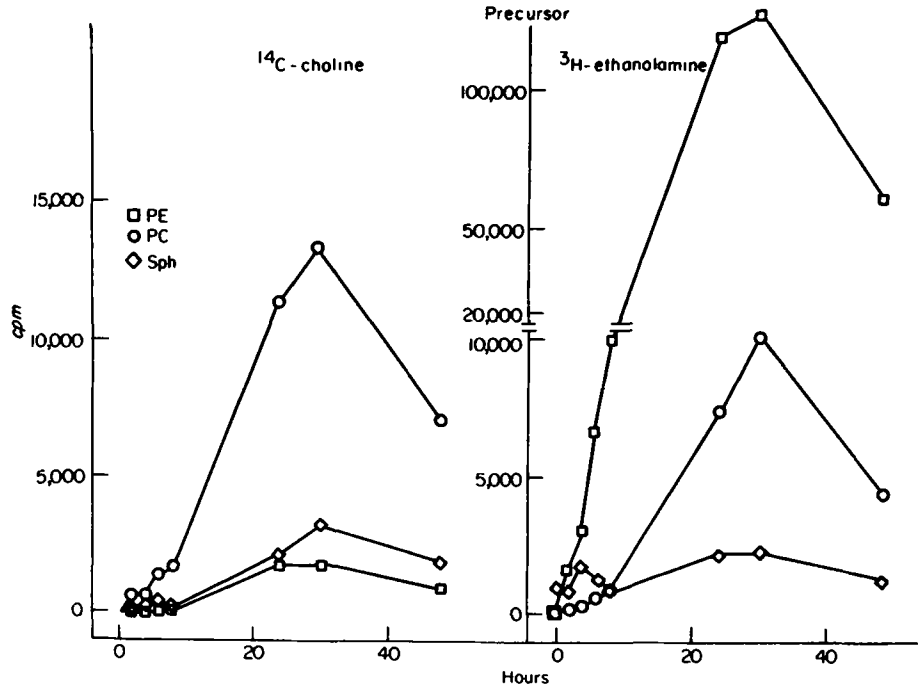

FIG. 8. Analysis of continuous uptake of [^{14}C] choline (5 μCi/ml; Sp. Act. = 4.2 mCi/mmole) and [^{3}H] ethanolamine (10 μCi/ml; Sp. Act. = 3.8 mCi/mmole) into phospholipids and total lipids extracted from stationary phase (100% sheeted monolayers) HEF cells. See legend under Figure 1 for Experimental Procedures.

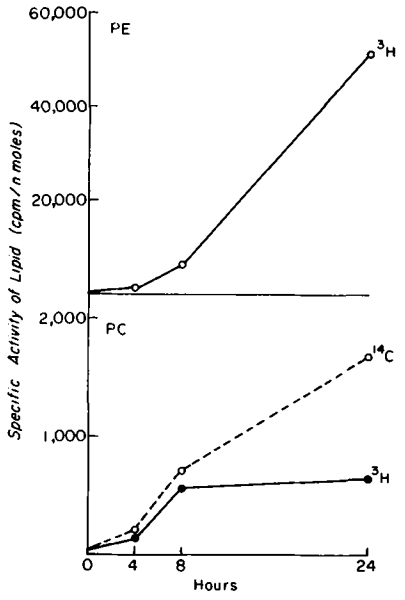

FIG. 9. The specific activities of the phosphatidyl ethanolamine and phosphatidyl choline were determined in an experiment similar to that described in Figure 8 except that each lipid was extracted and the lipid phosphorus quantitated as described in Experimental Procedures.

was due to other metabolic fates such as oxidation.) Figure 5 shows that most of both labeled precursors were incorporated into PC (panel a) and TG (panel b). Similar to the findings presented in Figure 3, very little radiolabeled free fatty acid was recovered which indicates a very rapid incorporation of exogenous fatty acid into cellular glycerides. This is further demonstrated by Figure 6 which compares the specific radioactivities of the free fatty acids. At no time did the specific activity of the cellular fatty acid reach that of the extracellular pool. When compared with individual phosphoglycerides (Fig. 7), we found that, based on the total acyl content, the cellular fatty acid pool had the higher specific radioactivity. However, when we consider that only about 20% of the fatty acid was [^{3}H] palmitate and 7-17% was [^{14}C], the specific activities of the individual fatty acid in the phosphoglycerides was actually higher than that of the cellular fatty acid pool. These data suggest that there is a pool of fatty acid derived from exogenous sources that does not mix with the total cellular pool. This exogenous pool is preferentially incorporated into phosphoglycerides which accounts for its high specific radioactivity. Comparison of the three phospholipid classes studied demonstrates that their synthesis (measured by increase in specific

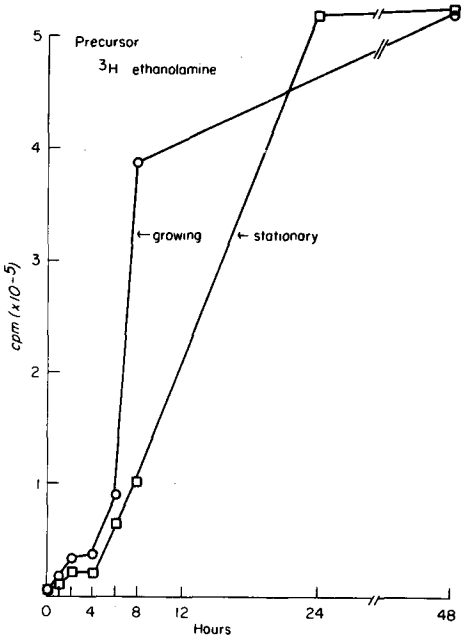

FIG. 10. Analysis of continuous uptake of [^{3}H] ethanolamine (10 μCi/ml; Sp. Act. = 3.8 mCi/mmole) into growing phase (the cells were about 75% sheeted monolayers) and stationary phase (100% sheeted monolayers) HEF cells. See legend under Figure 1 for Experimental Procedures.

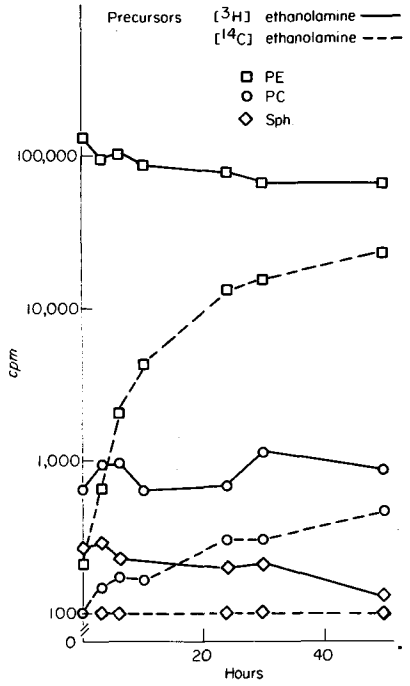

FIG. 11. Analysis of continuous uptake of ethanolamine into total phospholipids extracted from growing phase (about 75% sheeted monolayers) HEF cells. The cells were suspended in 2 ml growth medium supplemented with [^{3}H] ethanolamine (10 μCi/ml; Sp. Act. = 3.8 mCi/mmole) and seeded into 35 mm petri dishes. After 24 hr incubation at 37 C, the growth medium was aspirated, the cell monolayers were washed with 0.01 M Tris-saline, pH 7.4, and overlaid with 2 ml fresh growth medium supplemented with [^{14}C] ethanolamine (10 μCi/ml; Sp. Act. = 58 mCi/mmole). Incubation was continued at 37 C. At various times, some of the cultures were harvested and analyzed for uptake of the labeled precursors into phospholipids extracted from the HEF cells as described in Experimental Procedures.

radioactivity) paralleled their relative concentrations in the cells; namely, of the three, PC is the major lipid present, 40%; PE is intermediate, 35%; and PS + PI is least, 25%. It is of interest to note that the incorporation of radiolabeled fatty acid did not continue after the first 8 hr even though only 50% of the fatty acid pool was used. This would be the result of a cessation of phospholipid synthesis or the turnover being at a steady state that cannot be measured by this technique. The data presented in Fig. 8 are consistent with the latter interpretation.

The source of the base moiety of the lipid was studied by adding [^{3}H] ethanolamine and [^{14}C] choline (Fig. 8). In this experiment, it is possible to measure both the synthesis de novo of the major phospholipids, PC, PE, and Sph, as well as the methylation of PE which produces PC. We did not attempt to measure any intermediates in the methylation process. Surprisingly, the results with [^{14}C] choline (Fig. 8) showed an 8 hr delay before labeled Sph was detected. Some lag in the synthesis of [^{15}C] PC was seen, possibly due to the large pool of unlabeled choline in the culture medium. We do not know the pathway which leads to the formation of the small amount of [^{14}C] labeled PE (Fig. 8). After 30 hr, there was a marked decrease in [^{3}H] ethanolamine incorporated into PE and in PE which had been converted to PC (Fig. 8, right panel). These results were not seen in all experiments and might reflect some variation in the culture conditions of HEF cells. The incorporation of [^{3}H] ethanolamine into PE did not exhibit any lag whereas the conversion of PE to PC was delayed about 8 hr. These results would be expected of a precursor-product relationship. In a separate experiment, we determined the specific radioactivity of the PE and PC labeled with [^{3}H] ethanolamine and [^{14}C] choline (Fig. 9). When expressed in this fashion, it can be seen that the amount of lipid newly synthesized, relative to the amount of endogenous lipid, is quite small. This accounts for the initial low specific activity. The specific activity of the [^{14}C] choline in the medium

initially was 5 x 10^5 cpm/nmole. Since the maximum specific activity of [^{14}C] choline that was found was in the order of 2 x 10^3, we can conclude that very few of the phospholipid molecules are newly synthesized under these conditions. Similar calculations can be made with the [^{3}H] ethanolamine. In this experiment, there was a 1.25-fold increase in the amount of PE recovered from the cells after 24 hr of culture. The value for PC was 2.0. Results of experiments designed to compare [^{3}H] ethanolamine incorporation into growing phase cells with stationary phase cells showed that maximal rate of incorporation was delayed by several hours (Fig. 10). Presumably this was the result of difference in the pool size of endogenous ethanolamine in the two types of culture. We also found that the [^{14}C] methyl methione was incorporated into the PC of dividing cells. In this case, there was no lag period in the formation of PC as the preexisting PE served as substrate.

To determine the intracellular site and metabolic pathway utilized for HSV envelope phospholipid synthesis, experiments need to be designed to differentiate preexisting membrane lipids from membrane lipids synthesized de novo after virus infection. Toward this end, experiments were done to determine if it is possible to differentiate between preexisting and de novo synthesized membrane lipids by sequential labeling with [^{3}H] ethanolamine and [^{14}C] ethanolamine (Fig. 11). We chose labeled ethanolamine as a precursor since two major metabolic events can be measured; first, PE synthesis de novo, and second, methylation of PE. Growing phase cells were incubated in the presence of [^{3}H] ethanolamine. After 24 hr at 37 C, when the cells had reached a stationary phase of growth (confluent monolayers), the cultures were washed and incubation was continued in the presence of [^{14}C] ethanolamine for various times before extraction of the lipids (Fig. 9). Results showed that during a 48 hr period, there was a net loss of about 50% of the [^{3}H] labeled lipids (1.3 x 10^5 to 6.5 x 10^4 cpm). During this same period, there was incorporation of 2.3 x 10^4 cpm of [^{14}C] ethanolamine in labeled lipids. Based on the relative specific activities of the labeled ethanolamines ([^{3}H] specific activity was 65 times greater than [^{14}C]), it appears as if more of the new [^{14}C] PE was formed in the stationary phase cells as compared to PE measured in growth phase cells.

In summary, results of this investigation demonstrated the major metabolic pathways of lipid synthesis in HEF cells as well as some of their compositional characteristics. The cells can (a) use both glycerol and glucose for the glycerol backbone of the lipid, (b) use saturated and unsaturated fatty acids and can incorporate acetate into the fatty acids which comprise phosphoglycerides, and (c) synthesize PE and PC de novo and methylate PE to form PC. Further, the cells can synthesize lipids in both the stationary and growing phases although the latter is more rapid. Since we are able to sequentially label lipids with [^{3}H] and [^{14}C] ethanolamine, we believe this experimental design will be suitable for future studies on the genetic origin of intracellular site of synthesis and metabolic pathway required for the formation of specific lipid components needed for biogenesis of the herpesvirus envelope.

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Deca-2, 4, 6-Trienoic Acid, a New Conjugated Fatty Acid, Isolated from the Latex of *Euphorbia pulcherrima* Willd.

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ABSTRACT

From the latex of *Euphorbia pulcherrima*, a new conjugated trienoic fatty acid was isolated and identified as deca-*trans*-2, *trans*-4, *cis*-6-trienoic acid. Four other isomers of this acid were also present as minor components. The acids were esterified with triterpenols.

INTRODUCTION

Unsaturated fatty acids with three conjugated double bonds are mainly known from the seed oils of several plant species (1). All these trienoic acids have 18 carbon atoms, and occur as components of glycerides. Of the 8 possible geometric isomers, only the *cis-trans-cis, cis-trans-trans,* and *trans-trans-cis* isomers are found in the seed oils (1). A trienoic acid with a branched chain has been isolated from the leaves of *Eremophila oppositifolia* R. Br. (Myoporaceae) (2). This acid has 17 carbon atoms and a *trans-trans-trans*-triene linkage. Conjugated trienoic acids with another number of carbon atoms are not found in plants, in contrast to the conjugated dienoic (3) and acetylenic (4) acids.

The main compounds occurring in many plant lattices are fatty acid esters of tetra- or pentacyclic triterpenols (5-6). Usually the esterified acids are acetic acid or mixtures of long-chain fatty acids (5-8); sometimes cinnamic acid is the main esterified acid. Hitherto, the acidic components of latex lipids have not been studied in detail.

In the present work, the latex of *Euphorbia pulcherrima* Willd. (Euphorbiaceae), the Poinsettia, was investigated. This latex contains triterpenols and triterpene esters (6,9). The main part of the esterified acids appeared to consist of an unknown conjugated decatrienoic acid. Conjugated fatty acids esterified with triterpenols have not been isolated before from plants. The present paper describes the isolation and identification of this constituent.

EXPERIMENTAL PROCEDURES

Isolation and Preparation of Derivaties of the Fatty Acids

Latex (2-3 ml) collected from severed petioles was suspended in 0.1 M $MgCl_2$ and extracted with petroleum ether (bp 40-60 C). After evaporation of the solvent, the residue was dissolved in 2 ml benzene and saponified with 8 ml 0.5 N NaOH-methanol in a closed tube for 45 min at 100 C. After cooling and dilution with 10 ml water, the nonsaponifiable compounds were extracted with petroleum ether. The saponification mixture was then acidified to pH 1 with 2 N HCl. The free fatty acids were extracted with petroleum ether, evaporated to dryness and methylated with 3 ml 0.5 N HCl-methanol for one hour at 80 C. Saturated NaCl solution (6 ml) was added, and the methyl esters were extracted with pentane.

Purification of the triterpene esters by column chromatography and saponification-methylation with NaOH and boron trifluoride were carried out as described previously (10).

Adducts with maleic anhydride: Methyl decatrienoate (2 mg), maleic anhydride (5 mg), and benzene (1 ml) were heated in a sealed tube for 5 hr at 100 C; 2 ml petroleum ether was added and the mixture was evaporated to dryness after washing with water. The residue was dissolved in CS_2 and the adduct crystallized at 0 C.

Partial reduction with hydrazine: Methyl decatrienoate (2 mg), absolute ethanol (3 ml), and hydrazine hydrate (0.5 ml, 100%) were heated in an open tube for 5 hr at 50-55 C, while air was bubbled through. After dilution with 6 ml water, the products were extracted with pentane.

Oxidative splitting of the methyl esters (1-2 mg) by permanganate-periodate (11) was carried out. The reaction mixtures (5 ml) contained 50% t-butanol. At the end of the reaction (8 hr at 25 C), 10 mg NaOH was added and the mixture was evaporated on a steam bath. The residue was dissolved in 5 ml water, acidified with 2 N H_2SO_4 to pH 1, and the volatile free fatty acids were extracted with ethyl ether.

All treatments were carried out under nitrogen and at room temperature unless otherwise mentioned.

Gas Liquid Chromatography (GLC)

GLC of fatty acid methyl esters was performed with a Becker 409 instrument with FID utilizing coiled 1.8 m x 4 mm ID glass columns

TABLE I

Composition and Equivalent Chian Length (ECL) Values of the Main Fatty Acids of *E. pulcherrima* Latex

Methyl ester	Wt %[a]		ECL Values	
	HCl	BF_3	Apiezon-L 150 C	DEGS 185 C
8:0	10.4		8	8
10:0	12.0		10	10
A	10.3	4.3	11.35	13.78
B	1.9	2.3	11.46	
C	8.6	10.7	11.69	15.37
D	2.6	4.5	11.94	15.79
E	39.6	41.2	12.07	16.06
Rest	14.6			

[a]Area percentages by gas liquid chromatography (GLC) of the fatty acid methyl esters obtained after methylation with either BF_3 or HCl, determined on Apiezon-L column.

packed with either 25% DEGS or 10% Apiezon-L on Chromosorb-W, AW, 80-100 mesh. The DEGS column was operated at 185 C, the Apiezon-L column at 150 C. The nitrogen flow rates were 25 and 30 ml/min, respectively. Fractions were collected from the Apiezon-L column via a splitter inserted in the outlet of the column.

The free volatile fatty acids were determined on a 15% PPGS column (12) operating at 120 C and a nitrogen flow rate of 25 ml/min.

Spectroscopy

Infrared spectra were determined in CS_2 or CCl_4 on a Perkin-Elmer 257 spectrophotometer with NaCl plates.

Ultraviolet spectra were determined on a Beckman DB-25 spectrophotometer in cyclohexane.

Gas chromatography-mass spectrometry was carried out on a Jeol JMS-07 instrument operating at 70 eV, using a 3% SE-30 column.

RESULTS AND DISCUSSION

The lipid extracts of *E. pulcherrima* latex contain mainly triterpenols and triterpene esters. Rubber is a minor constituent of the latex. Thin layer chromatography (13) did not reveal the presence of other compounds in the extracts. The fatty acid methyl ester composition derived from the latex lipids was identical to that of the latex triterpene esters purified by column chromatography. This result implies that the acids obtained from a whole latex lipid extract were derived from triterpene esters.

Saponification conditions as described in Experimental Procedures were necessary to obtain complete hydrolysis of the triterpene esters. Decreasing the temperature to 60 C gave a saponification of only 25% of the triterpene esters. The fatty acid methyl ester composition was not altered by varying the saponification conditions.

Gas Liquid Chromatography

The fatty acid methyl ester mixture of *E. pulcherrima* latex was analyzed on the polar DEGS and the nonpolar Apiezon-L stationary phases. In Table I, the equivalent chain length (ECL) values and the weight ratios of the main fatty acids are given. Methyl octanoate and decanoate were present in high concentrations. These lower fatty acids are usually present in relatively small quantities (14).

Five compounds (A-E) could not be identified by comparison with known acids. After hydrogenation in methanol with platinum oxide, the compounds A-E were all converted into methyl decanoate. They consequently were unsaturated fatty acids with a straight carbon chain.

On the DEGS and the Apiezon-L columns, compounds A-E eluted after the corresponding saturated acid (Table I). On a nonpolar column, methylene interrupted unsaturated fatty acids elute before the saturated analogues. The presence of conjugated double bonds considerably increases the retention times on both polar and nonpolar columns (15). On nonpolar columns, all the geometric isomers of conjugated trienoic acids elute after the corresponding saturated acids (16). Compounds A-E were apparently conjugated fatty acids. They could be isolated as free acids by crystallization from pentane at -25 C.

The concentration of the conjugated fatty acids in the latex of *E. pulcherrima* determined on the Apiezon-L column with methyl

myristate as an internal standard was 2-3 mg/g fresh latex.

The conjugated fatty acid methyl ester fraction showed weight ratio differences (Table I), when they were methylated with either BF_3 or HCl. Quantitatively, the amount of conjugated fatty acid was not affected by the methylation procedures. Probably some bond migration took place during the preparation of the methyl esters under different circumstances.

Wolff and Miwa (16) demonstrated that extensive isomerization of trienes can occur during gas chromatographic analysis. Therefore, fractions of compounds A-E were collected and rechromatographed on the Apiezon-L column, but alterations of the trapped compounds were not observed during rechromatography.

During GLC of conjugated fatty acids on both polar and nonpolar columns, the *cis* isomers elute before the corresponding acids with *trans* configuration (15). Attempts to equilibrate the conjugated acids by treatment with iodine and ultraviolet light were rather unsuccessful. Such failures were also reported by Crombie (17) with deca-2, 4-dienoic acids and by Jefferies and Knox (2). In all these cases, the double bonds were in conjugation with the carboxyl group.

Oxidative splitting by von Rudloff's method (11) gave propanoic acid of the compounds A and B, and butanoic acid of the compounds C-E.

Gas Chromatography-Mass Spectrometry (GC-MS)

The conjugated fatty acid methyl esters were analyzed by GC-MS on a nonpolar SE-30 column. Mass spectra of A, B, C, and E were recorded. The fragmentation patterns of the compounds were appreciably different, but the m/e values were identical.

The molecular ion had m/e 180. The intense ion m/e 43 indicated a fragment $C_3H_7^+$. The peaks m/e 107 and 121 could be attributed to $CH_3(CH_2)_n(CH{=}CH)_3^+$, where n=1 and 2, respectively. The presence of three double bonds is further evident by comparing these mass spectra with those of ethyl deca-*trans*-2, *trans*-4, *cis*-7-trienoate (18). These results, combined with the data obtained by GLC, led to the conclusion that compounds A-E were isomers of methyl decatrienoate.

Infrared Absorption Data

Infrared absorption spectra were obtained for compounds A, B, C, and E. Compound D could not be collected in sufficient amounts by GLC to obtain suitable spectra for identification.

The infrared absorption spectra of compounds A and B were almost identical with that of methyl α-eleostearate (19). The infrared curve showed a strong band at 993 cm^{-1}, and a weaker one at 962 cm^{-1}. The carbonyl absorption at 1740 cm^{-1} indicated no conjugation with the double bonds. Oxidative splitting of the compounds A and B gave propanoic acid, showing that the triene grouping is 3, 5, 7. The purified amounts of compounds A and B were insufficient to obtain further evidence for identification by chemical treatments. Probably the structures of compounds A and B consist of the *trans-trans-cis* and *cis-trans-trans* isomers of methyl deca-3,5,7-trienoate.

Compound C was characterized by absorption maxima at 1719 (s), 1612 (w), 1176 (s), 1002 (s), 971 (s), and 820 (m) cm^{-1}. These data were in good agreement with those of methyl deca-*cis*-2, *trans*-4-dienoate (17). Conjugation of the double bonds with the carbonyl group is demonstrated by the absorptions at 1719, 1002, and 820 cm^{-1}. The latter is characteristic of a *cis* double bond conjugated with the carbonyl group. After treatment with maleic anhydride of a complete fatty acid methyl ester fraction from Poinsettia latex the compounds A, B, D, and E were converted for 80% into their adducts, except compound C, which remained unaltered compared with methyl decanoate by GLC. Therefore, the structure of compound C is suggested to be methyl deca-*cis*-2, *trans*-4, *cis*-6-trienoate.

Compound E was characterized by absorption maxima at 1720 (s), 1643 (w), 1620 (s), 1592 (2), 1255 (s), 1177 (m), 1140 (s), 1040 (m), 1002 (s), and 965 (w) cm^{-1}. Evidence about the position of the double bonds was given by the absorptions at 1720 and 1002 cm^{-1}. The shift of the carbonyl absorption to 1720 cm^{-1} indicated conjugation with the double bonds (20). The strong absorption at 1002 cm^{-1} points to a *trans* configuration in conjugation with a carboxyl group (17,21). The maximum at 965 cm^{-1} suggest that one of the double bonds may be *cis* (17,22).

With maleic anhydride an adduct of compound E was formed, providing evidence of a *trans-trans*-diene linkage (23). The mass spectrum of the adduct showed the expected molecular ion m/e 278. The infrared absorption spectrum of the adduct showed no *trans* unsaturation. Oxidative splitting of both compound E and its adduct gave butanoic acid. Compound E was partially hydrogenated by hydrazine. One product could be collected by GLC. Its infrared absorption spectrum was identical with that of methyl deca-*trans*-2,*trans*-4-dienoate (17).

From these results, the structure of com-

TABLE II

Ultraviolet Absorption Maxima for Methyl Decatrienoates (in Cyclohexane)

Compound	λ (mμ)			
A	258 (86)[a]	267 (max)	277 (72)	
B	258 (75)	267 (max)	278 (85)	300 (23)
C	286[b] (83)	297 (max)	310[b] (83)	
D	284[b] (74)	296 (max)	307 (82)	
E	283[b] (71)	294 (max)	305 (85)	

[a]Relative peak height abundance.
[b]Signifies an inflection.

pound E could be established as the methyl ester of deca-*trans*-2, *trans*-4, *cis*-6-trienoic acid.

Ultraviolet Absorption Data

In Table II, the ultraviolet absorption maxima of the conjugated acid methyl esters are shown. The spectra of the untreated latex lipid extracts and that of methyl deca-*trans*-2, *trans*-4, *cis*-6-trienoate (compound E) were almost identical, while the spectra of the fatty acid methyl ester mixtures showed some superposition of the spectra of compounds A and B. This means that compound E is the main natural conjugated fatty acid in the latex of the Poinsettia.

Compound E had ϵ=62500 (294 mμ). Its ultraviolet absorption superficially resembled that of α-parinaric acid (1), and occurred at longer wavelengths than the absorptions of the conjugated trienes not conjugated with a carboxyl group (1,24). A shift of the ultraviolet absorptions to longer wavelengths is also observed on the dienes, when the double bonds moved into conjugation with the carboxyl group.

Compounds A and B showed absorption maxima almost identical with the spectrum of β-eleostearic acid (25), suggestive for a *trans-trans-trans* configuration not conjugated with a carboxyl group. This is contrary to the infrared absorption data, which indicate *trans-trans-cis* configuration for the compounds A and B. These phenomena could not be explained by results from the literature.

Investigations of the lattices of other *Euphorbia* species revealed the presence of several other unknown conjugated fatty acids, which are now subject of further study.

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Free and Esterified Cholesterol in Developing Feline White Matter

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ABSTRACT

The content of free and esterified cholesterol was examined in the cervical spinal cord lateral funiculus and in the corpus callosum of the cat during postnatal development. The concentration of free cholesterol increased with development from 8 to 56 μg/mg in the lateral funiculus and from 4 to 37 μg/mg in the corpus callosum. The total content per specimen increased 130 and 70 times, respectively. The major part of the postnatal increase in the free cholesterol occurred late postnatally. The concentration of esterified cholesterol decreased with development from 0.96 to 0.07% and from 9.50 to 0.30% of total sterol in the lateral funiculus and corpus callosum, respectively. In both regions, the total content per specimen increased with maturation. Throughout development, the content of cholesteryl ester was higher in the corpus callosum than in the lateral funiculus. A transient increase in esterified cholesterol concentration was seen during the first postnatal days in the lateral funiculus and 3 weeks postnatally in the corpus callosum. The results suggest that most of the postnatal increase of free cholesterol in the white matter is related to a continued growth of established myelin sheaths and not to de novo myelination. The transient increase in concentration of esterified cholesterol in the early postnatal lateral funiculus coincides in time with a spontaneous myelin sheath disintegration. This supports the view that the ester peak may be primarily related to myelin breakdown rather than to myelin production. The significance of the high ester concentration in the neonatal corpus callosum and the ester peak seen during initial myelination remains obscure and calls for further developmental morphological studies.

INTRODUCTION

The presence of esterified cholesterol in the immature central nervous system has been demonstrated by several investigators (for references, see 1,2). Eto and Suzuki (1) pointed out that while the concentration of esterified cholesterol in the developing rat brain decreases with maturation, the total cholesteryl ester content per brain increases. Superimposed upon the general trend, a transient peak appears during the period of most active myelination (cf. 3). The appearance at early developmental stages of increased levels of esterified cholesterol has been related to the occurrence of OTAN-black and Marchipositive lipid droplets in developing white matter and has been interpreted by some workers as an importation and storage of lipids to be used for myelin synthesis (3-5). However, in the neonatal feline spinal cord white matter, degenerative features such as myelin sheath disintegration. glial cell death, and microglial phagocytes containing lamellated fragments, lipid droplets, and cellular debris have been observed (6-8). These findings suggested that a process resembling the nodalization process in developing peripheral nerves (9) might be operating in the central nervous system. In view of the fact that myelin degeneration is accompanied by an accumulation of esterified cholesterol (see 4), the transiently increased concentration of this compound in immature white matter might, instead, be accounted for by myelin breakdown.

Against this background and since available reports on the cholesteryl ester content of spinal cord white matter deal with other species than the cat, the present investigation was initiated. The principal aim was to obtain a measure of the content of free and esterified cholesterol in the postnatally developing lateral funiculus of the cervical spinal cord to supplement previous morphological studies in the same region. For comparison, specimens were also taken from the corpus callosum.

EXPERIMENTAL PROCEDURES

Thirty-three kittens, ranging in age from newborn to 6 months (Table II), and three adult cats (2-5 years old) were used. The anaesthetized (40 mg/kg BW Nembutal, IP) animals were exsanguinated by perfusion with Tyrode solution (1). The lateral funiculus of the cervical spinal cord was cut free, carefully blotted with a filter paper, and weighed. From most of the animals, the posterior half of the corpus callosum was also taken.

The pieces were immediately homogenized

TABLE I

Water Content in Cervical Spinal Cord Lateral Funiculus of the Cat During Postnatal Development

Age of animals	Number of animals	Percent water (SD)
1 day	6	87.9 (1.3)
3 days	3	85.6 (3.4)
5 days	5	84.9 (1.5)
1 week	3	82.6 (0.5)
2 weeks	4	83.0 (3.0)
3 weeks	4	80.8 (0.9)
4 weeks	6	76.7 (1.8)
7 weeks	4	73.6 (1.1)
2 months	5	71.8 (0.5)
3 months	4	69.3 (0.3)
Adult	5	65.4 (2.2)

in methanol (> 2 ml/100 mg tissue) using a glass homogenizer. After addition of two volumes of chloroform, the homogenates were extracted under nitrogen during at least 4 hr with 4 changes of chloroform-methanol (2:1) (10). All solvents were analytical grade. After filtering, the combined extracts were taken to dryness, redissolved in chloroform, filtered, and again taken to dryness. The dry extracts were dissolved in chloroform to a volume corresponding to 10-20 mg extracted material per ml. Samples of the crude extracts were placed on 6 x 100 mm columns with Unisil Silicic Acid (Clarkson Chemical Company, Camden, NJ). The neutral lipid fraction was eluted with 20 ml chloroform (11), evaporated, redissolved in a small amount of benzene-hexane (1:1), and placed on another Unisil column. Esterified cholesterol was eluted with 10 ml benzene-hexane (1:1) and the free cholesterol fraction was obtained with 20 ml chloroform (12). The purity of the separated fractions was checked by thin layer chromatography using 20 x 20 cm glass plates covered by a 0.25 mm layer of Silica Gel G (Merck AG, Darmstadt, Germany), activated at 105 C for 2 hr before use. The plates were developed in 1,2-dichlorethane and lipid spots were visualized with 0.0025% Rhodamin B.

The dried fractions of free and esterified cholesterol were redissolved in chloroform. Samples of this solution were diluted to give readings corresponding to between 0.25 and 2.5 μg cholesterol/ml after processing according to the fluorometric procedure described by Bondjers and Björkerud (13). The fluorescence of the final reaction mixture was measured in a Turner fluorometer using thoroughly cleaned 5 x 50 mm glass tubes. Commercial cholesterol and cholesteryl palmitate (Sigma Chem. Co., St. Louis, MO) were used as standards at concentrations of 0.25-2.5 μg cholesterol per ml final reaction mixture. In this interval, the increase in fluorescence with increasing cholesterol concentration was found to be linear. Since linear readings can be obtained in a concentration range from 4 ng to 2.5 μg cholesterol (13), the presented analysis is well above the lower sensitivity limit of the method.

The dry weight of adult and immature feline lateral funiculus white matter has not been reported elsewhere and, therefore, determinations were made on a separate material (Table I) by weighing before and after freeze-drying to constant weight. Dry weights of the corpus callosum are available in the literature (14,15).

RESULTS

Water in Lateral Funiculus

Lateral funiculus white matter from newborn animals had an average water content of 87.9% (Table I) and with development the water content decreased at a progressively slower rate. In the adult, the actual region of white matter contained 65.4 % water.

Free Cholesterol in Lateral Funiculus

At birth the average content of free cholesterol in the cervical lateral funiculus was 8.3 μg/mg wet weight (wet wt) and remained at about the same level during the first postnatal days (Table II). After 4 days, an increase in cholesterol was observed, which lasted beyond 6 months. In the adult, the content of free cholesterol was 56.5 μg/mg (Table II). The period of most rapid increase in cholesterol concentration occurred during the second half of the first postnatal month, but 66% of the entire postnatal increase in cholesterol concentration took place after the first postnatal month. On a dry weight basis, the content of free cholesterol increased 2.4 times between birth and adulthood from 68.6 to 163.3 μg/mg dry wt. Most of this increase (60.5%) occurred after the first month. The total content of cholesterol per specimen increased about 130 times with development and 95% of this increase occurred after the first postnatal month.

Esterified Cholesterol in Lateral Funiculus

The concentration of cholesteryl esters in lateral funiculus white matter decreased with development from an average level of 0.080 μg/mg wet wt at birth to around 0.026 μg/mg wet wt by 4 and 6 months (Table II). The major part of this decrease took place during the first 3 postnatal weeks. Between 6 months and the adult stage, the mean cholesteryl ester concentration increased to 0.042 μg/mg wet wt (Table

TABLE II

Content of Free and Esterified Cholesterol in Cervical Spinal Cord Lateral Funiculus and Corpus Callosum of the Cat During Postnatal Development

Age of animals			Mean content of free cholesterol		Mean content of esterified cholesterol	
Lateral funiculus	Number of animals	Mean specimen wet weight (mg)	μg/mg Wet weight (SD)	μg per Specimen	μg/mg Wet weight (SD)	μg per Specimen
0.1 day	5	35	8.3 (1.6)	288	0.080 (0.040)	2.7
4 days	3	35	7.5 (0.9)	265	0.097 (0.029)	3.4
9 days	5	62	11.4 (2.6)	709	0.051 (0.025)	3.2
14 days	4	62	15.0 (1.2)	929	0.068 (0.027)	4.2
3 weeks	6	72	14.2 (1.7)	1030	0.047 (0.016)	3.4
4 weeks	3	110	24.7 (2.3)	2720	0.040 (0.004)	4.4
4 months	3	496	43.7 (7.9)	21700	0.026 (0.012)	12.9
6 months	4	736	44.7 (1.3)	32800	0.026 (0.008)	19.1
Adult	3	685	56.5 (8.9)	38700	0.042 (0.007)	28.8
Corpus callosum						
0-1 day	3	9	4.0 (1.0)	37	0.38 (0.06)	3.5
4 days	3	11	5.4 (2.8)	60	0.23 (0.16)	2.5
14 days	3	14	7.6 (2.0)	104	0.23 (0.06)	3.2
3 weeks	3	15	5.7 (0.8)	87	0.25 (0.17)	3.8
4 weeks	3	30	11.4 (2.6)	343	0.13 (0.04)	3.9
6 months	3	43	26.7 (4.2)	1160	0.16 (0.11)	6.9
Adult	2	70	36.9 (8.1)	2600	0.11 (0.03)	7.8

II). The content of esterified cholesterol per mg dry weight decreased 5.5 times from 0.66 to 0.12 μg during development. This decrement was largely accomplished 1 month after birth. While the concentration decreased, the total content of esterified cholesterol in the cervical lateral funiculus specimens increased 10.6 times.

The ratio between the concentrations of esterified and free cholesterol in the spinal cord white matter (ester-free ratio) increased from about 0.010 to around 0.013 during the first postnatal days and then decreased with development to 0.0006 by 4 and 6 months attaining a value of 0.0007 in the adult (Fig. 1). Ninety percent of the postnatal reduction in ester-free ratio was accomplished by the age of 1 month.

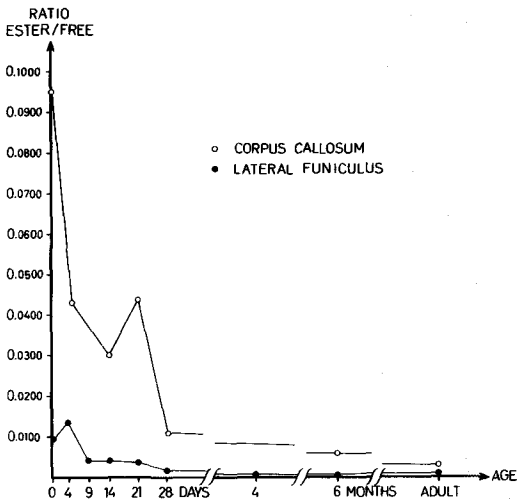

FIG. 1. Ratio between the content of esterified cholesterol and the content of free cholesterol (ester-free ratio) in the cervical lateral funiculus and the posterior half of the corpus callosum, plotted against age. Values were calculated from the data in Table I.

Free Cholesterol in Corpus Callosum

In the corpus callosum specimens, the mean level of free cholesterol was about 4 μg/mg wet wt at birth and 36.9 μg/mg wet wt in the adult (Table II). During the first postnatal month, the values showed considerable variation and there was no distinct increase until after 3 weeks. On a dry weight basis, using the values for water content given by Agrawal et al. (14,15), the cholesterol content increased 3.2 times from 49.4 μg/mg at birth to 159.0 μg/mg in the adult. The total content of free cholesterol per specimen increased some 70 times with development and 88% of this increase took place after the first postnatal month.

Esterified Cholesterol in Corpus Callosum

The highest cholesteryl ester level in the corpus callosum specimens was seen at birth (0.38 μg/mg), and in the adult the average concentration of esterified cholesterol was 0.11 μg/mg (Table II). A transient plateau appeared at 3 weeks. Considered per mg dry weight (14,15), the level of esterified cholesterol decreased 10 times with development from 4.69

to 0.47 μg in the corpus callosum specimens. The total content of cholesteryl ester per specimen increased 2.2x with development.

The ester-free ratio in the corpus callosum initially decreased from an average level of 0.095 at birth to 0.030 2 weeks later. Thereafter followed an increase to 0.044 by 3 weeks and then a rapid decrement ensued (Fig. 1). In 1-month-old kittens, the ratio was 0.011 and in the adult 0.003 (Fig. 1).

DISCUSSION

The changes in water content of the spinal cord lateral funiculus during the course of maturation followed a pattern similar to that previously observed in comparable regions of the feline and canine brain, although at a lower level (14,15).

The cholesterol values presently obtained from mature feline spinal cord white matter (56.5 μg/mg wet wt) agree with previous determinations of free cholesterol in the bovine spinal cord white matter (54-59 μg/mg wet wt) by Amaducci et al. (16). The level of free cholesterol in the adult feline corpus callosum (36.9 μg/mg wet wt) is lower than in unspecific cerebral white matter of the cat (44-46 μg/mg, 17) as well as in comparison with cerebral white matter of some other species (see 18 for references) but resembles the values presented for the human optic tract by Friede and Hu (19).

When considered on a wet weight basis, the mature lateral funiculus contains more cholesterol than the corpus callosum. On a dry weight basis, however, the two areas show a similar cholesterol concentration. This discrepancy may be explained by the difference in water content (65% in lateral funiculus, 77% in corpus callosum, 14,15), which most likely is related to the differing fiber spectra (16,20,21).

Comparisons between the neonatal concentration of free cholesterol in lateral funiculus white matter or corpus callosum with values obtained from corresponding regions in other species, e.g., chick (22,23), lamb (24), piglet (25), rat (26), rabbit (27,28), monkey (29), and man (30,31), reveal certain differences which are in agreement with differences in the general central nervous system maturity at birth (see 32).

On a wet weight basis, the concentration of cholesterol increased 6.8 times with development in the spinal cord and 9.2 times in the corpus callosum. On a dry weight basis, the increase was 2.4 and 3.2 times in the feline lateral funiculus and corpus callosum (14,15), respectively. In both cases, the period of most rapid increment in cholesterol concentration coincides with an intense de novo myelination (20,21). The change in cholesterol concentration may thus reflect the myelination of previously unmyelinated fibres.

Considering instead the total increase in free cholesterol per specimen, a different picture is obtained. In the spinal cord specimens, the average content of free cholesterol increased 130 times with development and 93% of this increase took place after the first postnatal month. In the corpus callosum, the amount of cholesterol per specimen increased 70 times and 88% of this increase occurred after the age of 1 month. After the early period of initial myelination, the subsequent developmental increase in free cholesterol per specimen should mainly reflect a continuing accretion of myelin in relation to a given population of nerve fibers. It thus seems that the main mass of myelin is produced late postnatally by growth of established myelin sheaths (33).

In agreement with previous findings in other species, the present results show that in feline white matter cholesteryl esters constitute a higher proportion of the total cholesterol during the early postnatal period than later in development. In the lateral funiculus specimens, the content of esterified cholesterol showed an early peak at 4 days and then decreased, mainly during the first postnatal month. Low values were seen at 4 and 6 months, while the adult level was somewhat higher, approaching that in 1-month-old kittens. Neonatally about 1% of the total lateral funiculus white matter cholesterol was in ester form and the corresponding adult figure was 0.07%.

The level of esterified cholesterol in neonatal lateral funiculus resembles that in the immature feline brain where 0.4-0.9% of the total cholesterol is esterified at ages varying between 1 day and 2 weeks (2). In developing and mature human whole brain, Rouser and Yamamoto (34) observed higher values of esterified cholesterol, in comparison with the feline lateral funiculus. Adams and Davison (3) reported very high levels of esterified cholesterol in the spinal cord of newborn infants. The whole brain of developing and mature rats presents lower values at all stages (1). In the spinal cord of postnatal lambs and piglets, Patterson et al. (24,25) did not find any esterified cholesterol at all.

In earlier reports (6,7), light- and electron-microscopical features suggesting a disintegration of myelin sheaths of early myelinating fibers were observed in the cervical lateral funiculus of kittens during the first 3 postnatal

weeks. In the same region of white matter, the present results show an increased occurrence of esterified cholesterol during the same time period. In addition, the presently found postnatal decrease in cholesterol ester content in the cervical lateral funiculus follows a course that is very similar to the decrease in occurrence of clusters of Marchi-positive lipid droplets in the same region (8). This lends support to the view that the cholesteryl esters constituting the ester peak might result from a spontaneous breakdown of myelin sheaths.

The ester values presently found in the neonatal feline corpus callosum (0.20-0.40 μg/mg wet wt) were higher than those in the spinal cord white matter and, although varying in size, this difference persisted throughout development. Similarly, Svennerholm (31) observed that the ester content of the corpus callosum exceeds that of some other cerebral white matter regions in the newborn human brain. The cholesteryl ester levels found in the developing feline corpus callosum are generally comparable with observations by other workers in developing human white matter (17,31,34,35) and in the developing chick brain (36), although for obvious reasons, the time schedules are different. However, the esterified cholesterol content in the corpus callosum of kittens and cats differs greatly from the levels observed in the immature and mature human corpus callosum by Adams and Davison (3,37) and is much higher than that in the developing rat brain (1).

In the corpus callosum, the ester peak at the end of the third postnatal week coincides in time with the formation of the first myelin sheaths (21). When myelin sheath formation is initiated, the increase in width of the corpus callosum has already been almost completed (38). Thus, if the initial myelination results in a complete ensheathment of the callosal fibers with formation of Ranvier nodes, the growing internodes would be able to elongate very little during development unless some internodes were eliminated. An internodal elimination with myelin sheath breakdown then appears to represent one possible explanation for the cholesterol ester peak in the 3-weeks-old corpus callosum (9). Such a mechanism does not explain, however, the high levels of ester cholesterol at birth, or throughout development the persisting pronounced difference in cholesterol ester content in the corpus callosum and the spinal cord white matter. This calls for morphological studies to supplement the chemical data.

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The Mass Spectrometry of Iso and Anteiso Monoenoic Fatty Acids

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ABSTRACT

The normal, iso, and anteiso Δ^8- and Δ^9-17:1 fatty acid methyl esters were synthesized and their electron impact-induced fragmentation was studied by mass spectrometry. The mass spectra of the preterminal branched monoenoic fatty acid methyl esters present characteristic fragment ions, now understood to be indicative of the position of the methyl group. These fragment ions are in the iso compound m/e 227 $[M-55]^+$, m/e 195 $[M-87]^+$, and m/e 177 $[M-105]^+$, while in the anteiso compound these fragments are shifted by 14 mass units to m/e 213, m/e 181, and m/e 163. The 15-D-iso Δ^8- and Δ^9-17:1 methyl esters were synthesized because the characteristic fragment ions in the methyl branched compounds indicated a key role of the tertiary hydrogen atom in the rearrangement process. A fragmentation mechanism consisting of a double bond migration triggered by the tertiary hydrogen and an allylic cleavage assuming a displacement mechanism is proposed.

INTRODUCTION

Iso and anteiso monoenoic fatty acids are members of Nature's fatty acid museum of curiosities. They have been recognized in the phospholipids of *Bacillus cereus* (1) with a Δ^5 double bond, in excretion products of *Myxococcus xanthus* (2) (an ubiquitous inhabitant of soil and plant debris) with a Δ^{11} double bond, in two antibiotics (3) and in mammalian skin lipids (4), e.g., isoΔ^6-16:1 and anteiso Δ^6-17:1 in vernix caseosa and adult human skin lipids (5). They were recently found as minor constitutents among the fatty acids of a marine diatomaceous ooze (6), which is kept anaerobic by the sulfate reductive activity of *Desulfovibrio desulfuricans.* Their presence in the marine deposit feeding polychaete, *Arenicola marina,* indicated by literature data (7), could be confirmed. *A. marina* contains small amounts of branched chain monoenoic fatty acids in its sterol esters, triglycerides, alkenylglycerides, and phospholipids (J.J. Boon, unpublished results). The presence of the iso and anteiso monoenoic fatty acids in both the marine sediment and the marine deposit feeder was understood by the finding of these acids in *D. desulfuricans,* a bacterium which was shown to contain about 35% of this fatty acid type among its fatty acids (8). Their iso and anteiso monoenoic fatty acids are likely, therefore, to play an increasing role as marker molecules in ecochemical and geochemical studies.

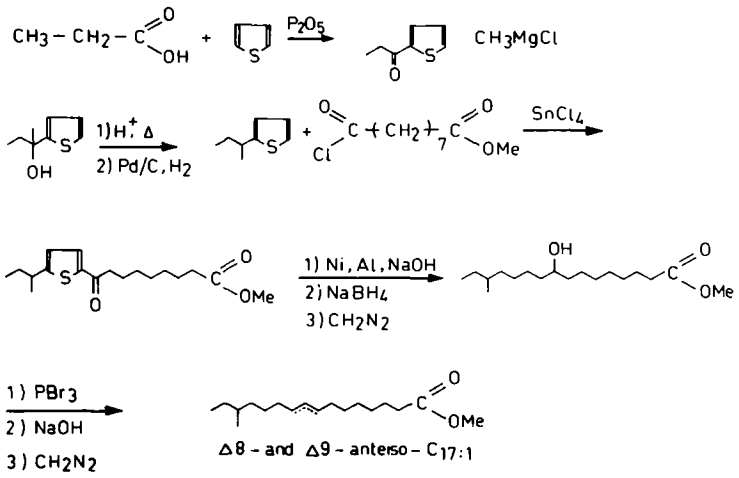
The identity of the iso and anteiso Δ^9-17:1 methyl esters isolated from *D. desulfuricans* is confirmed by mass spectra of the authentic standard compounds presented in this study. These mass spectra showed fragment ions which were thought to be related to the position of the methyl group in the alkyl chain.

This study presents a fragmentation mechanism for preterminal branched monoenoic fatty acid methyl esters. Data have been obtained by exact mass measurements, direct analysis of daughter ions (DADI) (9), and high voltage scans on the authentic normal, iso, and anteiso Δ^8- and Δ^9-17:1 methyl esters. As a support for the working hypothesis of the fragmentation mechanism, the 15-D-iso Δ^8- and Δ^9-17:1 methyl esters were synthesized. The combined presence of a double bond and a preterminal methyl group in fatty acid methyl esters induces fragmentations which have not been previously shown to occur in fatty acids (10). The resulting characteristic fragmentation pattern makes identification quite easy even in complex mixtures of fatty acids where they are present as minor components.

METHODS

Synthesis of the Standard Compounds

The synthetic route used in the synthesis of the normal-, iso-, anteiso-, and 15-D-iso Δ^8- and Δ^9-17:1 methyl esters is based, in principle, on a chain extension method, which consists of coupling suitable thienyl alkanes to half ester chlorides of the desired chain length (11) (Scheme I). In this way, the 1-thienyl-butane (12) and 1-thienyl-2-methyl-propane (12) are the precursors for the normal and iso fatty acid methyl ester. For the anteiso structure, (2-thienyl)-propan-1-one (12) was methylated by a Grignard reaction and reduced to the 2-(2-thienyl)-butane. The precursor for the 15-D-iso



Scheme I

Δ^8- and Δ^9-17:1 methyl ester was prepared from 1-[2-methyl-(2-thienyl)]-propan-1-one by incubation in D_2O at 120 C in the presence of anhydrous K_2CO_3. The resulting 2-D-2-methyl-1-(2-thienyl)-propan-1-one was transformed into the corresponding dithioketal. Chain extension was achieved by reaction of the four prepared thiophene derivatives with 8-chloroformyl-octanoic acid (12) (Merck Chemical Co., Darmstadt, Germany).

The dithioketal group in the deuterated compound was catalytically reduced with Raney nickel. The thiophene group was reduced with nickel aluminum (12) The intermediate 9-keto methyl esters were reduced with $NaBH_4$ in ethanol into the corresponding 9-hydroxy methyl esters. Treatment with PBr_3, debromination with aqueous NaOH, and esterification with diazomethane yielded mixtures of the Δ^8 and Δ^9 isomers.

All reaction products in the course of the synthetic procedures were identified by means of infrared, nuclear magnetic resonance, and gas chromatography-mass spectrometry (GC-MS). The methyl esters were purified from the synthetic mixtures by preparative thin layer chromatography (TLC) on silica gel using CCl_4 as the eluant. The purified deutero methyl ester was further purified by TLC on $AgNO_3$ impregnated silica gel by development with hexane-diethyl ether (95:5, v/v). The purity of the deutero compound was found to be 97% by appropriate mass chromatography on the Hitachi MS 52-Nova system.

Mass Spectral Measurements

Mass spectra of the normal, iso, and anteiso 17:1 methyl ester (Figs. 1A, 1B, and 1D) were obtained on a Varian-MAT 111 GC-MS system, equipped with a 30 m (ID 0.25 mm) stainless steel open tubular column coated with OV-101. Helium was used as carrier gas. Mass spectra were obtained at 70 eV. Metastable analysis (DADI and high voltage scans) was carried out using the MAT 311A mass spectrometer on the iso and anteiso 17:1 methyl ester. High resolution data were obtained from the 15-D-iso 17:1 methyl ester by direct probe analysis on a modified AEI MS 902 double focusing mass spectrometer (dynamic resolution 10,000). Data acquisition was achieved using LOGOS II, employing the XDS Sigma 7 computer (13). The mass spectrum of the 15-D-iso 17:1 methyl ester was obtained on a Hitachi MS 52 GC-MS system, equipped with a 42 m glass SCOT column (ID 0.75 mm) coated with OV-101. Helium was used as carrier gas. Mass spectra were obtained at 70 eV by cyclic scanning (scan time 3.19 sec, rescan time 1.59 sec) over a mass range of m/e 20-617. Data acquisition was achieved using a NOVA computer with a digitization rate of 12.9 kHz.

The cited mass spectrum of 2-methyl-1-pentadecene, used as reference mass spectrum, is present as mass spectrum no. 21 in the file of Mass Spectrometry Data Center, Aldermaston.

RESULTS AND DISCUSSION

The identity of a number of iso and anteiso monoenoic fatty acids present in *D. desulfuricans* (8) could be confirmed by the mass spectra of the authentic iso and anteiso 17:1 fatty acid methyl esters (Figs. 1B, 1D). Although the authentic standards are, in fact, mixtures of two double bond isomers (Δ^8 and Δ^9) as a consequence of the synthetic procedure, this has

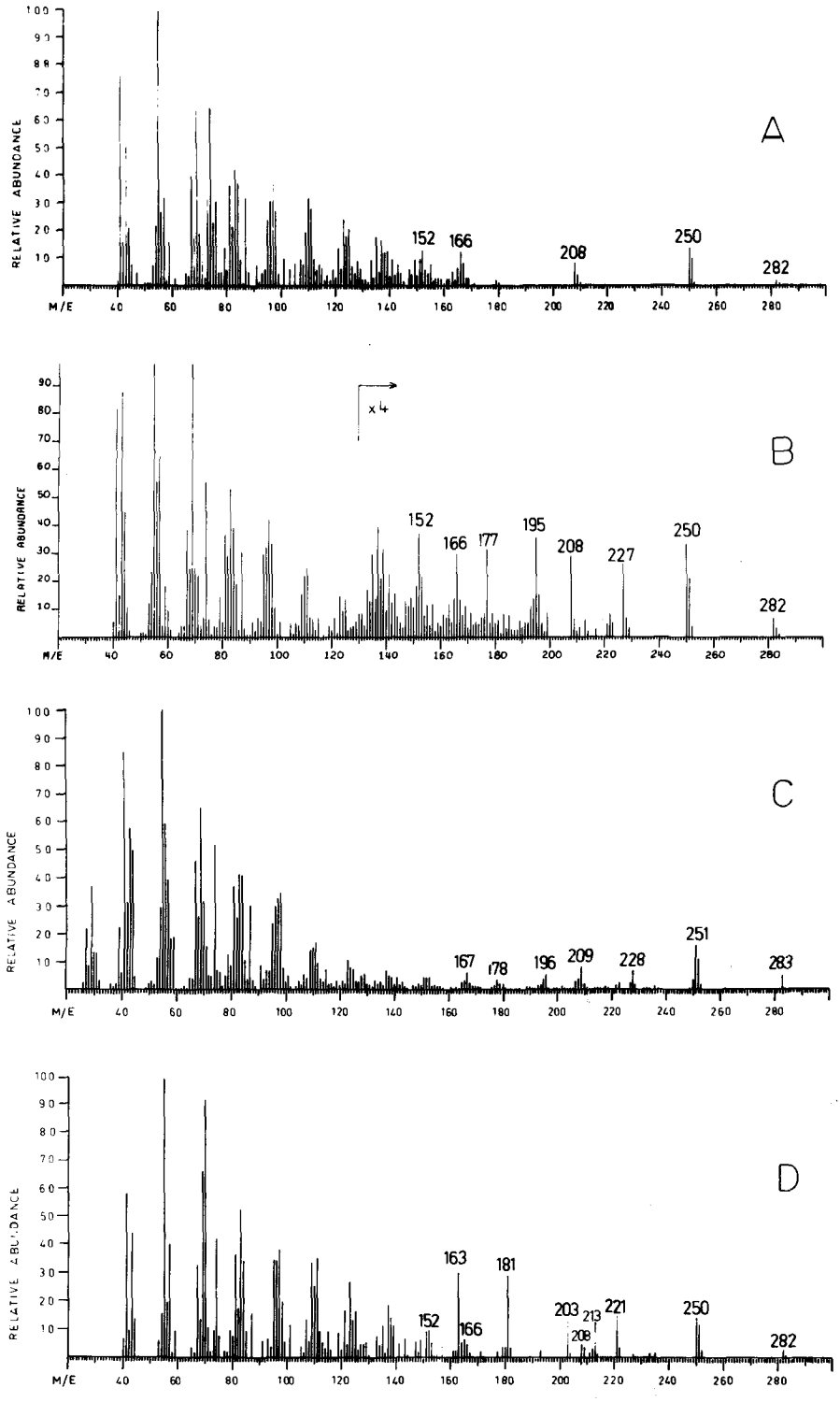


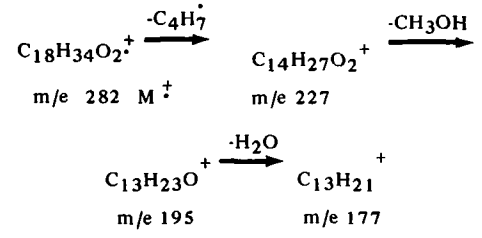
FIG. 1. Mass spectrum of the authentic normal, iso, 15-D-iso, and anteiso 17:1 fatty acid methyl ester. A = normal, B = iso, C = 15-D-iso, and D = anteiso compound.

no influence on the mass spectrum.

The mass spectrum of the iso Δ^9-17:1 fatty acid methyl ester of *D. desulfuricans* [double bond position located by GC-MS after derivatization to the corresponding di-trimethylsilyloxy derivative (8)] is similar to the mass spectrum of the iso Δ^{11}-17:1 fatty acid methyl ester of *M. xanthus* [double bond position located by GC-MS of reaction products obtained by oxidative degradation (2)]. Both are identical to the standard compound mixture of iso Δ^8- and Δ^9-17:1 fatty acid methyl esters (Fig. 1B). Moreover, double bonds are easily isomerized in the ion source of a mass spectrometer during analysis of unsaturated methyl esters (10).

The mass spectra of the normal, iso, and anteiso 17:1 fatty acid methyl ester, presented in Figures 1A, 1B, and 1D, show characteristic differences. The higher end of the mass spectrum of the straight chain compound (Fig. 1A) shows, besides the molecular ion, only major peaks at m/e 251, 250, 209, and 208. These peaks correspond to losses of 31, 32, 73, and 74 mass units from the molecular ion, losses which are generally observed for monoenoic fatty acid methyl esters (except those substituted at carbon atom two) (10,14).

In the mass spectrum of the iso 17:1 methyl ester (Fig. 1B), additional peaks at higher masses are found at m/e 227, 195, and 177. From metastable ions, DADI and high voltage scans, as well as exact mass measurements, it is concluded that these fragment ion peaks are formed predominantly by the following fragmentations:



DADI and high voltage scans carried out on the anteiso 17:1 methyl ester indicate similar fragmentations: loss of C_5H_9 (69 amu), followed by loss of methanol and water. The fragment ion peaks are shifted by 14 mass units to m/e 213, 181, and 163 (compare Fig. 1D).

The difference between the mass spectral behavior of the iso and anteiso 17:1 fatty acid methyl ester strongly suggests that, in the first step of the electron impact induced fragmentation, four C-atoms for iso and five for anteiso are lost from the end of the alkyl chain after migration of two hydrogen atoms. The spectrum of the 15-D-iso 17:1 fatty acid methyl ester (Fig. 1C) shows that one of these hydrogens originates from position C_{15}, the tertiary carbon atom. The fragment ion peaks at m/e 227 and m/e 195 in Figure 1B are shifted mainly to m/e 228 and m/e 196 in the deuterated compound. Apart from preterminal branching, the presence of a double bond is essential, since loss of 55 and 69 amu is not observed in mass spectra of the saturated iso and anteiso fatty acid methyl esters, respectively (14-16).

These findings and considerations may be rationalized by a migration of the double bond towards the end of the alkyl chain triggered by a rearrangement of the hydrogen on the tertiary carbon atom. An allylic cleavage then results in the loss of $C_4H_7^{\bullet}$ from the molecular ion of iso 17:1 and $C_5H_9^{\bullet}$ from the molecular ion of the anteiso 17:1 fatty acid methyl ester, if one assumes a displacement mechanism (14) shown in Scheme II. Allylic cleavage as such is not expected to result in the loss of $C_4H_7^{\bullet}$ or $C_5H_9^{\bullet}$. The mass spectrum of 2-methyl-1-pentadecene does not show a peak of some intensity for an ion $[M-55]^+$. The relative abundance for this fragment ion is less than 0.1% after correction for isotopic contributions.

ACKNOWLEDGMENTS

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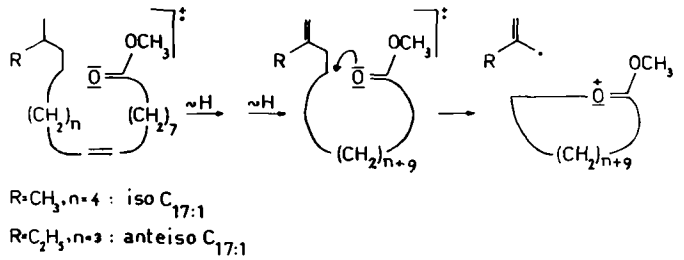

Scheme II

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Fatty Acid Composition at the 2-Position of Ether-Linked and Diacyl Ethanolamine and Choline Phosphoglycerides of Human Brain Tumors

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ABSTRACT

The acyl composition of ethanolamine and choline phosphoglycerides from a series of human brain tumors was determined and compared to that of normal human gray matter. Six glioblastomas, one astrocytoma, one oligodendroglioma, and one meningioma were analyzed. The total fatty acid composition of ethanolamine phosphoglycerides generally had a higher percentage of 18:1, 18:2ω6, and 22:5ω3 and a lower percentage of 22:6ω3 than that of normal gray matter. Choline phosphoglycerides from the tumors also contained a higher than normal percentage of 18:2ω6. Separate analysis of the acyl groups at the 2 position of the diacyl and ether-linked components of the phosphoglycerides revealed that the diacyl component of ethanolamine phosphoglyceride from the tumors had lower than normal amount of 22:6ω3 and a higher than normal amount of 18:1 and 18:2ω6. The acyl composition of ether-linked ethanolamine phosphoglycerides genearally contained a higher percentage of 20:4ω6 and a lower percentage of 18:1 compared to the corresponding fraction from normal gray matter. The astrocytoma analyzed had fatty acid profiles similar to those of the control with the exception of a greater 18:2ω6 content. These data demonstrate that the composition of the acyl moiety at the 2 position of diacyl and ether-linked phosphoglycerides of brain tumors differs from the corresponding component from normal gray matter and that the ether-linked ethanolamine phosphoglycerides provide an important pool of polyunsaturated fatty acids from brain tumor phospholipids.

INTRODUCTION

The lipids of human brain tumors have been the subject of numerous investigations since the first extensive study performed by Brante in 1949 (1), and a number of compositional differences in relation to normal brain have been found (see Ref. 2 for review). These include changes in sterol and sterol ester concentrations (1,3), alterations in neutral glyceride composition (4-6), and changes in phospholipid class distribution (4,5,7). The literature on brain tumors also contains numerous reports on the total acyl composition of the major phospholipid classes which is substantially different from that found in normal brain (8-12).

In addition to changes in the major lipid classes, brain tumors may also have altered composition of ether-linked glycerolipids (12,13) which are present in normal mammalian brain primarily as alkenyl acyl glycerophosphorylethanolamine (GPE). Little is known of the acyl components of ether-linked phospholipids in brain tumors, although studies in normal brain of the fatty acid moieties of plasmalogens have shown that they differ from their diacyl counterpart in composition (14) and metabolism (15). Because of these differences and the possible alterations in ether-linked lipid composition of brain tumors, it seemed likely that comparison of the fatty acid profiles of ether-linked phsopholipids with those of diacyl phospholipids from human brain tumors might aid in gaining a better understanding of the overall changes in fatty acid composition of brain tumor phospholipids. Therefore, in the present investigation, we have determined the acyl composition of ether-linked and diacyl components of major phospholipid classes of human brain tumors and compared them with those of normal adult human gray matter.

MATERIAL AND METHODS

The tumor and normal brain tissues used in this investigation were also included in a previous investigation of phospholipid composition (13). Human brain tumor samples were obtained during surgical procedures from Columbia University, the Medical University of South Carolina, and the Medical School of the University of North Carolina at Chapel Hill.

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The type of tumor represented by each sample was diagnosed by the laboratory furnishing the sample. The samples were kept in ice-cold saline solution until extraction procedures were initiated, generally within 1 hr after surgical removal. Normal brain specimens were obtained during autopsy within 12 hr after death. These tissues, if not analyzed immediately, were stored frozen. Gray matter was obtained from the brain samples by removing all visible traces of white matter.

Purified phospholipid classes were obtained from normal and tumor samples and subjected to hydrolysis by phospholipase C by the method of Waku et al. (16) as described previously (13). The diradylglycerols produced by the hydrolysis were separated into diacyl and ether-linked acyl glycerol fractions by preparative thin layer chromatography (TLC) (13). The ether-linked acyl glyceride fraction was then hydrolyzed and extracted by the saponification procedure described by Blank et al. (17). The resulting free fatty acids were obtained by preparative TLC (diethyl ether-acetic acid, 100:0.5, v/v). This hydrolysis procedure did not result in the release of free aldehydes from alk-1-enyl groups present. The free fatty acids were esterified for gas liquid chromatography (GLC) analysis with boron trifluoride (18). The diacyl glycerol fraction, after removing aliquots for analysis of total fatty acid composition, was subjected to pancreatic lipase hydrolysis by the method of Van Golde and Van Deenen (19). Ten mg of pancreatic lipase that had been extensively extracted with ether prior to use was suspended in 1 ml 1.0 M Tris buffer, pH 8, and added to the diacyl glycerol suspended in 0.1 ml 22% $CaCl_2$ and 0.3 ml 0.1% solution of sodium deoxycholate. This mixture was shaken vigorously for 15 min at 40 C. At the end of the reaction period, the mixture was acidified with 0.5 ml 6 N HCl and the lipids were extracted (20). The resulting 2-acyl glycerol fraction was separated by TLC (hexane-diethyl ether-acetic acid, 60:40:1, v/v) and transesterified by the method of Metcalfe et. al. (18) with boron-trifluoride-methanol reagent. The methyl esters were separated by GLC on a 12 ft x 0.08 in. coiled glass column packed with 12% EGSS-Y on 100-200 mesh Gas Chrom Q (Applied Science Laboratories, Inc., State College, PA). The instrument was a Perkin-Elmer Model 900 equipped with dual hydrogen flame ionization detectors and a digital integrator. The column temperature was 195 C. Injection port and manifold temperatures were 230 C and 250 C, respectively. Peaks were identified by comparison of retention times with known standards and with the aid of cal-

TABLE I

Composition of Fatty Acids Located at the 2 Position in Diacyl Glycerophosphorylethanolamine (GPE) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal adult gray matter (4)	5.5 ±2.7	2.5 ± 1.8	1.4 ±0.6	14.4 ± 2.3	0.3 ±0.1	---	1.1 ±0.1	1.4 ±0.4	23.8 ±2.2	9.9 ± 1.1	5.3 ±0.4	0.3 ±0.1	33.7 ± 3.3
Glioblastomas (6)	15.7 ± 5.1	3.6 ± 1.3	9.1 ± 3.9	28.0[c] ±4.7	7.8[c] ± 2.4	0.8 ± 0.3	1.0 ±0.4	2.1 ±0.9	20.7 ±2.0	3.1[d] ±0.8	1.3[d] ±0.7	0.8 ±0.4	4.3[d] ±0.6
Astrocytoma (1)	2.8	0.7	1.0	18.2	3.6	---	0.8	3.8	32.4	8.2	4.9	1.5	21.6
Oligodendroglioma (1)	5.1	1.5	1.1	25.6	11.2	0.2	1.6	4.1	25.3	10.2	1.6	1.8	8.8
Meningioma (1)	3.5	1.0	1.4	44.8	7.2	---	0.4	1.8	36.4	1.0	0.6	---	1.3

[a]Values expressed as the mole percent of total fatty acids ± SEM.
[b]Number of individual samples in parentheses.
[c]Differs significantly from corresponding normal, $p<.05$.
[d]Differs significantly from corresponding normal, $p<.01$.

TABLE II

Composition of Fatty Acids in Intact Diacyl Glycerophosphorylethanolamine (GPE) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal gray matter (4)	14.8 ±3.5	4.7 ±1.7	32.3 ±2.7	11.9 ±0.8	0.8 ±0.2	0.9 ±0.5	2.1 ±1.0	2.0 ±1.1	10.6 ±0.7	4.8 ±1.0	1.6 ±0.3	0.3 ±0.2	12.7 ±1.7
Glioblastoma (6)	18.8 ±5.4	2.8 ±2.4	27.3 ±4.7	19.4 ±4.4	3.8 ±3.1	1.8 ±1.4	---	2.0 ±1.7	11.7 ±3.1	3.1 ±0.5	0.8 ±0.3	1.3 ±1.1	3.8[c] ±1.2
Astrocytoma (1)	9.3	1.8	30.5	15.3	2.1	1.0	1.3	2.7	16.1	4.7	3.6	0.9	9.6
Oligodendroglioma (1)	9.1	2.0	24.6	16.7	1.5	2.8	5.1	4.4	17.5	6.7	1.1	0.9	2.3
Meningioma (1)	10.4	---	27.8	24.6	0.7	1.0	2.5	2.7	22.5	2.4	0.9	1.2	0.9

[a]Values expressed as the mole percent of total fatty acids ± SEM.
[b]Number of individual samples in parentheses.
[c]Differs significantly from corresponding normal, $p<.05$.

culated equivalent chain lengths. The chain length compositions were determined and converted to mole percentage based upon the molecular weight ratios of the derivative to an internal standard (heptadecanoate).

RESULTS

Ethanolamine Phosphoglycerides

The composition of fatty acids found in the 2 position of diacyl GPE and in intact diacyl GPE is given in Tables I and II, respectively. Several differences between normal and tumor samples were evident – foremost of these being a sharp decrease in 22:6ω3 apparent in all the tumors analyzed with the exception of the astrocytoma. This drop in 22:6ω3 was particularly evident in the profiles of the fatty acids in the 2 position (Table I). Concentrations of other polyunsaturated fatty acids including 22:4ω6 and 22:5ω6 were also generally lower in the tumor fractions compared to normal values whereas the amounts of 18:1 and 18:2ω6 were higher (Table I). The major exception was the aastrocytoma, which had only moderately altered levels of these fatty acids.

The fatty acid profiles of total diacyl GPE from tumors and normal tissue reflect the same compositional differences (Table II). Also evident is the high level of 18:0 present in diacyl GEP from both normal and tumor tissue. This fatty acid is located primarily in the 1 position. From data in Tables I and II, it can be calculated that 18:0 comprises 45-60% of the fatty acids in the 1 position of diacyl GPE of both tumors and normal tissue. 16:0 (13-22%) and 18:1 (4-12%) are also major components.

Normal human brain gray matter and the human brain tumors containe; considerable amount of ether-linked ethanolamine phosphoglycerides which were isolated and the fatty acid composition determined. As with the diacyl counterpart, differences between the acyl distribution in the 2 position of the ether-linked ethanolamine phosphoglycerides from normal and tumor lipids were apparent (Table III). Most striking was the high percentage of 20:4ω6 in the ether-linked ethanolamine phosphoglycerides of the tumors which, with the exception of the astrocytoma, was twice that of normal. In contrast to the diacyl component, the percentage of 18:2ω6 was not significantly higher than normal in the tumors. The average level of 18:1 was decreased compared to normal in the glioblastomas and the oligodendroglioma (Table III), whereas the level of 22:5ω3 was generally increased (Table III). The percentage of 22:6ω3 in the ether-linked ethanol-

amine phosphoglycerides was not significantly different from the normal value in the group of glioblastomas and the astrocytoma, although it was lower in the oligodendroglioma and the meningioma (Table III).

From the above compositional analysis, it was apparent that the fatty acids at the 2 position of both alkenyl (and alkyl) acyl GPE and diacyl GPE from brain tumors had compositions different from their normal tissue counterpart. It was further apparent that the alterations in the acyl composition of the ether-linked ethanolamine phosphoglycerides were not the same as those of diacyl GPE. In order to emphasize further the differences between the changes in acyl groups located in the 2 position of alkyl and alkenyl acyl GPE and of diacyl GPE, the molar ratios of various fatty acids from the 2 position of both groups were calculated and compared. As with the compositional data, definite trends were evident. The 18:1 ratio decreased from ca. 1.6 in normal to 0.7 or lower in all types of tumors except for the astrocytoma, whereas the 20:4ω6 ratio generally increased from about 0.6 to about 1.4. The change in the 18:1 ratio was due primarily to a relative increase in 18:1 in diacyl GPE, but the increase in the 20:4ω6 ratio was largely a result of increased levels of 20:4ω6 in ether-linked ethanolamine phosphoglycerides (Tables I and III). The low 18:2ω6 ratio of the tumors (0.3 to 0.5) compared to normal (2.9) is an indication of the sharp rise in 18:2ω6 concentration in the diacyl component not observed in the corresponding ether-linked component. The divergence between the acyl component of the ether-linked and diacyl ethanolamine phosphoglycerides was evidenced further by the 22:6ω3 ratio, which was higher for most tumors (0.8 to 3.5) than for normal brain (0.4). This increase in the ratios reflects the sharp reduction in the concentration of 22:6ω3 in the diacyl component compared to only moderate changes in concentration in the acyl component of the ether-linked counterpart in the tumors.

The primary exception to the trends noted above was the astrocytoma, which generally had ratios similar to that for normal human brain gray matter ethanolamine phosphoglyceride.

Choline Phosphoglycerides

Results of the analysis of fatty acids located at the 2 position of diacyl glycerophosphorylcholine (GPC) from brain tumors and from normal brain gray matter are in Table IV. Several differences between the normal and tumor fatty acid patterns were evident. The percen-

TABLE III

Fatty Acid Composition of Alkyl and Alkenyl Acyl Glycerophosphorylethanolamine (GPE) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal adult gray matter (4)	8.9 ±2.8	4.6 ±2.2	5.0 ±1.9	22.0 ±3.2	1.8 ±1.1	---	5.1 ±0.5	1.1 ±0.1	15.0 ±2.4	18.3 ±4.7	1.5 ±0.3	0.7 ±0.1	13.8 ±4.0
Glioblastomas (6)	5.2 ±1.4	1.9 ±0.8	3.1 ±0.7	14.1[c] ±2.1	2.6 ±1.2	0.5 ±0.2	2.8[c] ±0.7	2.5 ±0.8	33.0[c] ±5.8	13.7 ±2.7	2.2[c] ±0.2	2.7[c] ±0.8	13.8 ±2.3
Astrocytoma (1)	3.1	1.0	5.7	21.9	1.3	0.3	2.6	2.1	26.7	14.9	2.8	1.3	15.5
Oligodendroglioma (1)	3.8	1.4	5.3	13.2	5.5	0.8	2.6	3.1	34.3	13.5	2.2	4.3	7.4
Meningioma (1)	3.0	0.6	1.2	27.4	1.8	1.0	2.0	1.5	54.1	2.5	0.9	0.3	1.6

[a]Values expressed as the mole percent of total fatty acids ± SEM.
[b]Number of individual samples in parentheses.
[c]Differs significantly from corresponding normal, $p<.05$.

TABLE IV

Composition of Fatty Acids Located at the 2 Position in Diacyl Glycerophosphorylcholine (GPC) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal gray matter (4)	50.8 ±6.1	1.4 ±1.4	9.9 ±3.7	28.6 ±5.2	1.6 ±0.3	0.3 ±0.1	0.5 ±0.1	0.6 ±0.1	3.9 ±0.8	0.6 ±0.1	0.2 ±0.1	---	1.2 ±0.3
Glioblastomas (6)	33.9[c] ±3.5	4.8[c] ±0.8	4.0 ±1.9	33.4 ±1.1	9.8[c] ±3.0	1.1 ±0.4	0.7 ±0.2	2.0[c] ±0.4	5.6 ±1.1	1.2 ±0.3	0.4 ±0.2	0.6 ±0.2	0.9 ±0.2
Astrocytoma (1)	35.1	5.8	0.6	19.4	16.4	0.7	0.8	3.6	12.8	1.0	0.4	0.4	1.6
Oligodendroglioma (1)	36.0	3.7	0.6	32.2	15.5	0.7	0.9	1.8	5.3	1.0	0.2	0.4	0.6
Meningioma (1)	17.9	1.5	0.4	45.1	15.0	0.7	0.7	2.0	14.6	0.5	---	0.3	0.3

[a]Values expressed as the mole percent of total fatty acids ± SEM.
[b]Number of individual samples in parentheses.
[c]Differs significantly from corresponding normal, $p<.05$.

tage of 16:0 was lower than in normal brain diacyl GPC in all the tumors analyzed with the exception of the meningioma. The levels of 16:1 and 18:1 in tumors were higher than in normal brain with the exception of the astrocytoma, which had a reduced percentage of 18:1. Most prominent was the high content of 18:2ω6 found in the 2 position of diacyl GPC from brain tumors (this fatty acid was present in only small amounts in normal brain tissue) (Table IV). Other polyunsaturated fatty acids including 20:3ω6 and 20:4ω6 were also increased in tumors but to a lesser extent.

The fatty acid composition of total diacyl GPC from tumors and normal brain (Table V) had relatively low levels of 18:0 compared to the composition of diacyl GPE (Table II). This is reflected in the calculated composition of the 1 position of diacyl GPC in which 16:0 (30-70%) and 18:1 (20-50%) are the major components. 18:0 made up only 5-20% of the total fatty acids in the 1 position of diacyl GPC.

The fatty acid profile of ether-linked choline phosphoglycerides including alkyl and alkenyl ethers from brain tumors was compared to that of ether-linked choline phosphoglycerides present in normal brain. No statistically significant differences were apparent although the percentages of 18:2ω6 and 20:4ω6 were slightly higher than normal in most of the tumors (Table VI).

DISCUSSION

Previous reports have established that the total acyl composition of phospholipid classes of brain tumors differs from that of normal human brain (8-12). The results of the present investigation of the fatty acid composition of diacyl and ether-linked phospholipids provide additional conformational evidence of the alterations. By using the proportion of ether-linked lipid present and reported previously (13), the overall composition of each of the two major phospholipid classes can be calculated. These calculated compositions indicate that, compared to normal brain, brain tumor ethanolamine phosphoglycerides have high levels of 18:1, 18:2ω6, and 20:4ω6 and low levels of 22:6ω3. Similarly, fatty acids of choline phosphoglycerides from the tumors generally have higher levels of 18:2ω6 and lower levels of 22:6ω3 than does normal gray matter. These results, therefore, are essentially in agreement with those previously reported for total fatty acid composition of individual phospholipid classes by Stein et al. (8) and White (11).

The above findings are characteristic of most

TABLE V

Composition of Fatty Acids in Intact Diacyl Glycerophosphorylcholine (GPC) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal gray matter (4)	46.9 ±1.4	2.3 ±0.8	9.8 ±1.2	28.5 ±1.5	2.0 ±0.1	0.5 ±0.4	0.7 ±0.4	0.9 ±0.1	4.5 ±1.0	0.9 ±0.1	0.4 ±0.2	---	1.4 ±0.3
Glioblastoma (6)	45.4 ±4.4	3.7 ±0.9	7.8 ±1.7	26.6 ±2.2	6.9 ±1.9	0.8 ±0.2	1.0 ±0.2	1.4 ±0.4	4.1 ±0.6	0.4 ±0.1	0.2 ±0.1	0.2 ±0.1	0.4 ±0.1
Astrocytoma (1)	31.3	3.7	8.5	33.3	9.7	0.5	1.1	2.4	4.7	0.9	0.5	0.6	1.1
Oligodendroglioma (1)	34.2	2.6	10.6	28.5	11.6	0.5	1.4	1.8	4.0	1.2	0.4	0.6	0.8
Meningioma (1)	43.4	---	9.8	31.6	8.8	---	0.6	0.8	3.9	0.2	---	---	---

[a]Values expressed as the mole percent of total fatty acids ± SEM.

[b]Number of individual samples in parentheses.

[c]Differs significantly from corresponding normal, $p<.05$.

TABLE VI

Composition of Fatty Acids Located at the 2 Position in Alkyl and Alkenyl Acyl Glycerophosphorylcholine (GPC) from Human Brain Tumors and from Normal Brain[a]

Sample[b]	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3ω6	20:4ω6	22:4ω6	22:5ω6	22:5ω3	22:6ω3
Normal gray matter (4)	38.1 ±3.8	2.3 ±0.8	11.5 ±0.8	28.6 ±3.2	2.5 ±0.2	0.6 ±0.4	1.6 ±0.5	1.3 ±0.1	7.9 ±0.7	1.6 ±0.5	0.4 ±0.2	0.1 ±0.1	2.9 ±1.4
Glioblastoma (6)	38.5 ±3.1	3.1 ±0.4	10.3 ±1.5	26.7 ±5.4	5.9 ±2.3	0.7 ±0.3	1.1 ±0.1	1.9 ±0.4	8.9 ±2.8	1.0 ±0.3	---	0.6 ±0.2	0.9 ±0.1
Astrocytoma (1)	22.1	---	8.4	20.3	6.9	2.0	1.6	9.8	19.6	3.4	1.5	1.9	2.4
Oligodendroglioma (1)	34.4	---	11.8	29.4	6.0	---	1.6	1.7	10.1	2.1	---	0.5	1.0
Meningioma (1)	26.2	---	19.4	32.5	4.6	1.0	1.7	1.8	9.8	0.8	---	0.2	---

[a]Values expressed as the mole percent of total fatty acids ± SEM.

[b]Number of individual samples in parentheses.

tumors analyzed. However, the astrocytoma, which was of low grade malignancy and considered slow growing, displayed patterns similar to those of the control with the exception of high content of 18:2ω6. Although no firm conclusion can be drawn on the basis of the analysis of one sample, these results suggest that at least some of the changes in fatty acid composition observed may be related to rapid growth and cell proliferation rather than to changes fundamental to transformation and malignancy. The fatty acid profiles of the phospholipids of human brain tumors are, in fact, in some ways similar to those of normal human immature brain (10). Both the tumors and immature brain have a lower proportion of 22:6ω3 and a higher proportion of 18:1 than mature brain. However, the increased proportion of 18:2 ω6 and 20:4ω6 found in ethanolamine phosphoglyceride and 18:2ω6 in choline phosphoglyceride of brain tumors is not found in normal human featal brain (10) suggesting that these differences may in fact be related to transformation and malignancy.

In addition to confirming previous reports of the altered fatty acid composition of total ethanolamine and choline phosphoglycerides of human brain tumors, the present investigation demonstrates that the acyl composition of the ether-linked phospholipids differs substantially from that of their diacyl counterparts, both in normal brain and in the tumors. Furthermore, the changes in the overall fatty acid composition of the tumor phospholipids relative to normal brain are the result of the combination of changes, some in divergent directions, in the acyl distribution pattern of the ether-linked and diacyl phospholipids. Perhaps the most significant of these changes, both in terms of consistency and possible physiological effects, are those on arachidonate and 22:6ω3 distribution. That ether-linked ethanolamine phosphoglycerides of the tumors generally had a higher relative concentration of these polyunsaturated fatty acids than did their diacyl counterpart indicates that ether-linked phospholipids may serve for the neoplastic cell as an important source of polyunsaturated fatty acids. The possibility that this may be true in general for pathological tissue with altered lipid metabolism is supported by the findings of Wykle et al. (21) who demonstrated that arachidonate is preferentially retained in the ethanolamine plasmalogens of rat testes during essential fatty acid deficiency. However, regardless of whatever function ether-linked lipids might have in neoplastic tissue, the present investigation has demonstrated that the acyl moiety of the ether-linked phospholipids of human brain tumors differs in composition from its diacyl counterpart. As has been previously shown with normal mammalian brain (14), the importance of analyzing the acyl components of ether-linked phospholipids separately for more meaningful evaluation of tumor phosphoglyceride composition is apparent.

ACKNOWLEDGMENTS

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The Effect of Cerulenin on Sterol Biosynthesis in *Saccharomyces cerevisiae*

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ABSTRACT

Cerulenin specifically inhibited fatty acid biosynthesis in *Saccharomyces cerevisiae* without having an effect on sterol formation. Ergosterol was not required for cell growth in the presence of cerulenin (1 μg/ml). The addition of fatty acids to the growth medium reduced the amount of ergosterol formed by 45%; further addition of cerulenin to the media had no effect on the amount of ergosterol synthesized by the cells. The incorporation of ^3H from ^3H_2O into ergosterol was not affected by cerulenin whereas incorporation into fatty acids was inhibited by 90%.

INTRODUCTION

Cerulenin, an antibiotic isolated from the culture filtrate of *Cepharlosporium caerulens*, inhibited the growth of several different fungi, bacteria and yeast-type fungi (1,2). Subsequently, it was found that cerulenin inhibited the incorporation of [^{14}C]acetate into fatty acids and sterols in yeast (3,4). The mechanism whereby cerulenin inhibits fatty acid biosynthesis in *Escherichia coli* has been shown to be alkylation of a cysteinyl sulfhydryl of β-ketoacyl-ACP synthetase (5). However, the mechanism for inhibition of sterol synthesis is less clear cut. [^{14}C]Acetyl CoA but not [^{14}C]mevalonate or [^{14}C]HMG CoA incorporation into ergosterol was inhibited by cerulenin in cell-free extracts from *Saccharomyces cerevisiae* suggesting that the enzyme, HMG CoA synthetase, was the site of inhibition (6). These *in vitro* experiments were not confirmed in intact cells since the inhibition of cell growth by cerulenin could be reversed simply by addition of fatty acids without sterols (3). This would suggest that sterol synthesis was not affected by cerulenin in growing cells. In addition, Rottem and Barile (7) have shown that cerulenin inhibition of *Acholeplasna laidlawii* was not accompanied by inhibition of carotenoid formation, which was, in fact, stimulated. This suggestive evidence prompted a reinvestigation of the effect of cerulenin on sterol biosynthesis in growing cultures of *S. cerevisiae* since the earlier experiments were carried out with resting cells.

MATERIALS AND METHODS

The growth of yeast was carried out essentially as previously described (8). An inoculum of yeast cells was added to 50 ml of medium (1%) and cerulenin (prepared by Irving Putter of the Merck Research Laboratory, Rahway, NJ) was added from a 10 mg/ml stock solution in dimethyl sulfoxide. Fatty acids and ergosterol were added as indicated to the incubations at zero time from a 16.6 mg/ml stock solution in ethanol containing 10% Tween-80. The cells were grown to late log phase and harvested by filtering with type HA millipore filters. Lipids were extracted from the cells with 80% ethanol as described earlier (8). After addition of 5N KOH and heating of the solution for 1 hr at 90 C, the nonsaponifiable fraction was extracted with two 5 ml portions of petroleum ether. The basic solution was acidified with HCl, and the saponifiable fraction also was extracted with petroleum ether in a similar manner. Radioactivity in each of the lipid fractions was determined by thin layer

TABLE I

Effect of Added Lipids of Cerulenin Inhibition of Yeast Growth[a]

Cerulenin (μg/ml)	Palmitate + Oleate[b]	Ergosterol[c]	Growth (Klett units)
0	–	–	305
0	+	–	320
0	+	+	330
1.0	–	–	20
5.0	–	–	20
20.0	–	–	20
1.0	–	+	45
1.0	+	–	275
5.0	+	+	280
10.0	+	–	220
10.0	+	+	200
20.0	+	–	240
20.0	+	+	195

[a]Yeast grown in Yeast Nitrogen Base (DIFCO) plus 2% glucose.

[b]Palmitate and oleate at a final concentration of 0.1 mg/ml each.

[c]Ergosterol–0.06 mg/ml.

TABLE II

Effect of Cerulenin on Ergosterol and Fatty Acid Biosynthesis in Yeast[a]

Addition[b]	Cell growth (Klett units)	Sterols[c]		Fatty acids[c]	
		mg	cpm x 10^3[d]	mg	cpm x 10^3[d]
1. None	320	0.162	2.12	1.08	25.20
2. Plamitate + Oleate	315	0.090	1.04	1.28	11.00
3. Cerulenin + Palmitate + Oleate	315	0.112	1.37	1.12	1.04
4. Cerulenin	21	–	–	–	–

[a]Yeast grown in Yeast Nitrogen Base (DIFCO) plus 2% glucose.

[b]Palmitate and oleate at a final concentration of 0.1 mg/ml each.

[c]Results expressed as mg/100 mg of yeast dry weight.

[d]^3H_2O added to the medium at a concentration of 62 μc/ml.

chromatography of a portion of the nonsaponifiable and saponifiable extracts on Silica Gel G. The plates were developed in petroleum ether-diethyl ether-acetic acid (75:25:1) and visualized in an iodine chamber. The spots which corresponded to authentic oleic acid and ergosterol were scraped into scintillation vials and Aquasol 2 (New England Nuclear, Boston, MA) was added for counting.

Quantitative determination of the various lipids was carried out by gas chromatography of the extracts. A portion of the nonsaponifiable extract was taken to dryness, 1 ml of BSTFA-pyridine (2:1) was added, and the solution was heated to 60 C for 5 min. Chromatography of the samples was carried out on a 3% OV-1 column at 260 C. Methyl esters of fatty acid fractions were prepared with diazomethane and the esters chromatographed on a 10%-SP•2340 column (Supelco, Bellefonte, PA). The amount of ergosterol and fatty acids present in the samples was determined by integration of the peaks and comparison with appropriate standards.

RESULTS AND DISCUSSION

Table I describes the growth of yeast in the presence of cerulenin and various lipids. At 1 μg/ml, cerulenin completely inhibited the growth of the cells, and this inhibition was almost completely reversed by the addition of palmitate plus oleate. Although palmitate but not oleate was effective in reversing cerulenin inhibition (data not shown), the mixture of these fatty acids was usually more effective than palmitate alone. Ergosterol was not required with the fatty acids for reversal of inhibition even at a cerulenin concentration as high as 20 μg/ml, and it did not enhance cell growth over that seen with fatty acids alone.

The effect of cerulenin on the amount of lipids in yeast and the incorporation of ^3H into lipids from ^3H_2O is shown in Table II. The total amount of ergosterol (identified by gas chromatography) found in the yeast incubated with fatty acids was considerably less than that found in the absence of fatty acids. However, there was no additional reduction when cerulenin was added to the media. In fact, the amount of ergosterol in the yeast showed a small increase in the presence of cerulenin over that in the control with palmitate and oleate.

The incorporation of tritium from ^3H_2O into ergosterol was also reduced in the presence of fatty acids, but this decrease was probably due to the decrease in the amount of ergosterol synthesized since the specific activity was similar in all three incubations (1.30, 1.20, and 0.97 x 10^3 cpm/mg ergosterol in incubations 1, 2, and 3, respectively).

On the other hand, cerulenin almost completely inhibited fatty acid biosynthesis. Although the amount of fatty acids found in the yeast was similar in all three incubations, the addition of cerulenin inhibited the incorporation of tritium into fatty acids by 90% as compared to the cells grown with fatty acids alone. Fatty acids in the media also inhibited the incorporation of radioactivity from ^3H_2O by about 50%.

These experiments confirm the inhibition of fatty acid biosynthesis by cerulenin (1-5,9). However, the antibiotic had no effect on sterol synthesis over and above that seen with fatty acids alone. In fact, cerulenin stimulated the synthesis of sterols in a manner similar to the stimulation of carotenoid formation in *A. laidlawii* (7). The most obvious explanation for the stimulation of sterol formation in *S. cerevisiae* and *A. laidlawii* by cerulenin is the shunting of some of the excess acetyl CoA from the blocked fatty acid pathway to sterol biosynthesis.

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Effect of Substrate Polarity on the Activity of Soybean Lipoxygenase Isoenzymes

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ABSTRACT

In order to characterize the several isoenzymes of soybeans, they were examined with respect to the effect of the polar nature of the substrate. In general, lipoxygenase-1 was most active when presented with charged substrates such as the anionic form of linoleic acid or of potassium linoleyl sulfate, whereas lipoxygenase-2 and -3 preferred nonpolar substrates such as unionized linoleic acid, methyl linoleate, linoleyl methane sulfonate, 10,13-nonadecadieneamine, or linoleyl acetate. Linoleyl sulfate, which has been advanced as an excellent readily soluble substrate for lipoxygenase, was indeed the best substrate found for lipoxygenase-1. Lipoxygenase-2 and -3 were, by contrast, totally inactive against this substrate. The favorable response of linoleic acid to lipoxygenase-2 and -3 at pH 6.8 was ascribed to the anomalously high pK_a value of linoleic acid compared to that of short chain carboxylic acids. The pH-activity profile obtained with lipoxygenase acting on linoleyl sulfate (which was charged at all pH values examined) was shifted to lower pH values compared to the linoleic acid activity profile. The effect of changing from the charged to the uncharged substrate, when tested against lipoxygenase-1, was to increase the K_m by an order of magnitude.

INTRODUCTION

Lipoxygenase (EC 1.13.11.12), an iron-containing dioxygenase present in many plants, catalyzes the peroxidation of polyunsaturated fatty acids containing the *cis*-1,*cis*-4-pentadiene moiety (1). Three electrophoretically pure isoenzymes of lipoxygenase have been isolated from soybeans (2,3). These differ with respect to amino acid content, pH optima, positional specificity for oxygenation, and secondary product formation (2-5). We have examined the behavior of the three isoenzymes against a variety of linoleic acid derivatives and find that the enzymes differ markedly among each other with respect to their preference for the ionic or polar nature of the linoleic acid derivative.

MATERIALS AND METHODS

Enzyme Purification and Assay

The isoenzymes of soybean lipoxygenase were purified by the procedure of Christopher et al. (2,3) from soybeans of Amsoy variety (1975 crop). Lipoxygenase activities were determined using a Clark oxygen electrode (Yellow Springs) in a Gilson Medical Electronics Oxygraph, Model KM, as previously described (2,3). The reaction vessel had a volume of 1.5 ml. One unit of enzyme activity corresponds to the consumption of 1 μmole of O_2 per min. Assays were conducted at 15 C in 0.2 *M* phosphate buffer, pH 6.8, for lipoxygenase-2 and -3 and in 0.2 *M* borate buffer, pH 9.0, for lipoxygenase-1 unless otherwise indicated.

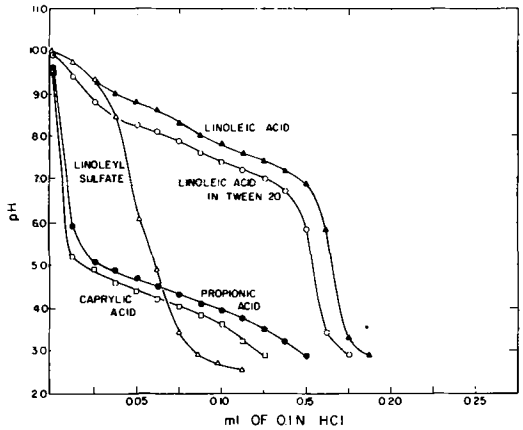

FIG. 1. Titration curves of linoleic acid and the potassium salt of linoleyl sulfate. All acids (0.1 *N*) were made alkaline with NaOH and titrated with 0.1 *N* HCl in a Radiometer Titrigraph. Linoleic acid was prepared in Tween 20 (-○-○-○-) or in 95% ethanol (-▲-▲-▲-). The potassium salt of linoleyl sulfate was prepared as an aqueous solution (-△-△-△-). Propionic acid (-●-●-●-) and caprylic acid (-□-□-□-) are shown for comparison.

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TABLE I

Isoenzyme Reaction Velocities with Linoleic Acid Derivatives

Substrate[a]	Velocity, μmole O_2/min/mg protein					
	Lipoxygenase-1		Lipoxygenase-2		Lipoxygenase-3	
	pH 9.0		pH 6.8		pH 6.8	
Linoleic acid	154		89		4	
Methyl linoleate	16		46		8	
Linoleyl alcohol	34		25		4	
Linoleyl methane sulfonate	27		160		7	
10,13-Nonadecadiene nitrile	11		58		7	
Linoleyl chloride	97		62		5	
Linoleyl acetate	9		35		3	
	pH 6.8	pH 9.0	pH 6.8	pH 9.0	pH 6.8	pH 9.0
Linoleic acid in Tween	4	157	32	<1	57	<1
Potassium linoleyl sulfate (aqueous)	146	216	<1	<1	<1	<1
10,13-Nonadecadieneamine	56	80	21	<1	<1	<1

[a]All assays at 15 C. All substrates prepared at a final concentration of 6.67×10^{-4} *M* in ethanol unless otherwise indicated. pH 9.0 assay buffer was 0.2 *M* borate; pH 6.8 assay buffer was 0.2 *M* phosphate.

Protein concentrations were calculated from absorbance values at 280 nm. Measurement of the dry weight of purified lipoxygenase gave a value of 0.7 mg protein per ml per absorbance unit for all three isoenzymes.

Substrates

Potassium linoleyl sulfate was prepared from linoleyl alcohol by the procedure of Allen (6). The white solid gave a single spot on a thin layer chromatogram (Silica Gel G) developed in hexane-ether-acetic acid (60:40:1). 10,13-Nonadecadieneamine was prepared from 10,13-nonadecadiene nitrile by the method of Yoon and Brown (7) using aluminum hydride as reductant. The formation of 10,13-nonadecadieneamine was confirmed by infrared spectroscopy. All other lipids (purity >99%) were purchased from Nu-Chek Prep (Elysian, MN). Solutions of substrates were prepared in either 95% ethanol or in Tween 20 by a modification of the method of Surrey (8).

pH Profile and Determination of Kinetic Constants

The activity of lipoxygenase-1 towards linoleic acid and linoleyl sulfate as a function of pH was determined at constant ionic strength (0.23). Acetate buffer was employed at pH 6.5 and lower; phosphate, pH 6.5-8.0; borate, pH 8.0 and higher. Experimentally indistinguishable values were obtained where buffers overlapped.

Kinetic constants were calculated on a Hewlett-Packard Model 9825A computor programmed to present Lineweaver-Burke plots based on linear regressions.

pK_a Determination

Apparent pK_a values of fatty acids were determined on a Radiometer Titrigraph, Model SBR3, by adding an excess of 1 *N* NaOH to the acid (0.1 *N*) and back titrating the solution with 0.1 *N* HCl.

RESULTS AND DISCUSSION

Apparent pK_a of Linoleic Acid

The pK_a of fatty acids with chain lengths of C_2-C_9 is reported to be between 4.82-4.95 (9). The apparent pK_a of linoleic acid was determined in a Radiometer Titrigraph by back titrating with 0.1 *N* HCl (Fig. 1). An unexpectedly high apparent pK_a of about 7.9 was observed under these conditions. The small chain fatty acids, propionic acid and caprylic acid, were found to have pK_a's in the expected range when determined in this same system.

Isoenzyme Variation with Derivatives of Linoleic Acid

A wide variation of response of the three isoenzymes for the various derivatives of linoleic acid was found (Table I). Lipoxygenase-1 was most active with linoleic acid and linoleyl sulfate. At the optimum pH reported for lipoxygenase-1 of 9.0, both substrates would be ionized. 10,13-Nonadecadieneamine was about 50% as active as linoleic acid as a substrate. Since the reported pK_a for alkyl amines is 10-11 (10), the 10,13-nonadecadieneamine would be expected to be predominately positively charged at pH 9.0. The nonpolar derivatives, methyl linoleate and linoleyl acetate, were poor substrates. When linoleyl

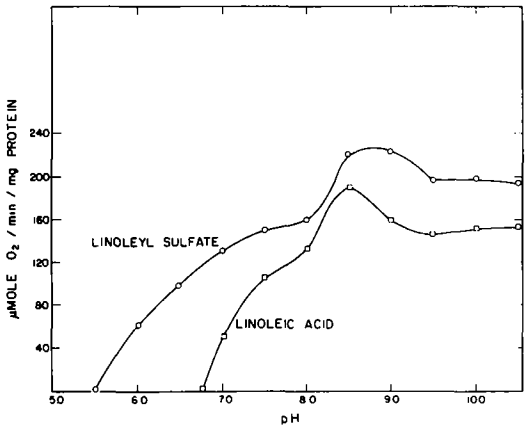

FIG. 2. pH profile of lipoxygenase-1 with linoleyl sulfate and linoleic acid. Reaction velocities were determined as μmoles of oxygen consumed per min per mg protein in the oxygraph. Buffers were prepared at constant ionic strength. Acetate buffer was used at pH 6.5 and below, phosphate pH 6.5-8.0 and borate pH 8.0 and above.

chloride was tested, the reaction rate decreased before either oxygen or substrate was completely consumed, suggesting that the enzyme was being inactivated.

Lipoxygenase-2, on the other hand, was most active with nonpolar substrates. At pH 6.8, the optimum for the enzyme, linoleic acid is predominately in the unionized form. Lipoxygenase-2 was totally inactive against linoleyl sulfate. The methane sulfonate was the best among the substrates tested.

Lipoxygenase-3 was significantly active only against linoleic acid at pH 6.8, apparently favoring the unionized state of the substrate. Like lipoxygenase-2, the enzyme was inactive against the sulfate.

The possibility that isoenzymes of lipoxygenase differ in their response to the polarity of substrates was first proposed by Koch et al. in 1958 (11). Employing two partially purified fractions of soybean extract, they proposed the existence of two isoenzymes based on the variation of their activities against linoleic acid and trilinolein. In 1970, Christopher et al. (2) reported that a second isoenzyme of soybean lipoxygenase differed from the original Theorell lipoxygenase (12) (lipoxygenase-1) in exhibiting a preference for methyl linoleate or trilinolein, as opposed to linoleic acid. Any physiological significance of these differences remains to be established.

The three isoenzymes also differed in their behavior with respect to the nature of the dispersal of the linoleic acid. Lipoxygenase-1 had nearly identical activity with linoleic acid prepared in ethanol and Tween 20, whereas lipoxygenase-2 was nearly 3 times as active with linoleic acid prepared in ethanol as that prepared in Tween 20. Lipoxygenase-3, on the other hand, was over 10 times more active when the substrate was prepared in Tween 20.

pH Profile of Lipoxygenase-1

The ratio of the activity of lipoxygenase-1 with linoleic acid at pH 9.0 and pH 6.8 was ca. 40 whereas with linoleyl sulfate the ratio was closer to 1.5. This difference may be accounted for by the ionization state of the substrates. Linoleyl sulfate is ionized throughout the pH range tested while linoleic acid is predominately nonionized below its apparent pK_a of 7.9. For similar reasons, the pH profile of lipoxygenase-1 with linoleic acid and linoleyl sulfate should differ, reflecting the preference of the enzyme for the charged substrate. Indeed, as Figure 2 shows, the pH profile of the enzyme with linoleyl sulfate was broadened and extended to lower pH values as compared to the profile obtained with linoleic acid. The optimum pH value was nearly the same with both substrates.

Kinetic Constants of Lipoxygenase-1 with the Free Acid and the Sulfate

The K_m's of lipoxygenase-1 for linoleic acid and linoleyl sulfate were determined at pH 6.8 and pH 9.0 (Table II). At pH 9.0, where both substrates are negatively charged, the K_m's were similar. However, at pH 6.8, where the linoleic acid is predominately nonionized and the linoleyl sulfate ionized, the K_m for linoleic acid was nearly 10 times higher than that for linoleyl sulfate. The V_{max} with linoleyl sulfate at pH 9.0 was only 1.5 times greater than at pH

TABLE II

Comparison of Kinetic Parameters of Lipoxygenase-1 with Linoleic Acid and Linoleyl Sulfate

	K_m		V_{max} (oxygen μmole/min/mg protein)	
	pH 6.8	pH 9.0	pH 6.8	pH 9.0
Linoleic acid	4.9×10^{-4} M	8.5×10^{-5} M	55	169
Linoleyl sulfate	5.5×10^{-5} M	8.0×10^{-5} M	121	181

6.8, while with linoleic acid the ratio was closer to 3.0.

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Polygala virgata Seed Oil – A New Source of Acetotriglycerides[1]

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ABSTRACT

The seed oil of *Polygala virgata* (family Polygalaceae) contains 74% of monoacetotriglycerides, the first found in nature with the acetate group in position 2 of *sn*-glycerol. Naturally occurring triglycerides characterized previously all have the acetate at position 3. The configuration of the acetoglyceride from *P. virgata* was established by a combination of thin layer chromatography, optical rotatory dispersion, and nuclear magnetic resonance.

INTRODUCTION

The seed oil of *Monnina emarginata* (family Polygalaceae) contains a complex mixture of triglyceride components in which are incorporated some unusual oxygenated fatty acids (1-3). *S*-Coriolic (13*L*-hydroxy-*cis*-9,*trans*-11-octadecadienoic) acid is the predominant oxygenated acid in that oil, representing 30% of the total fatty acids (1). Examination of the seed oil of another polygalaceous species – *Polygala virgata* – revealed a relatively low content (<5%) of coriolic acid or other conjugated dienol. However, gas liquid chromatography (GLC), nuclear magnetic resonance (NMR), and infrared (IR) indicated an unusually high concentration of acetate groups. This paper describes the isolation and characterization of a unique ATG from *P. virgata* seed oil. (Throughout the remainder of this paper, ATG = acetotriglyceride; poly-ATG = ATG from *Polygala virgata* seed oil; poly-ATGH = hydrogenated poly-ATG; cel-ATG = ATG from *Celastrus orbiculatus* seed oil; egg-ATGH = ATG from hydrogenated egg yolk lecithin.)

METHODS AND MATERIALS

Extraction of Seed

Coarsely ground seeds of *P. virgata* were extracted 6 hr with petroleum ether (bp 33-51 C) by the Soxhlet procedure. Solvent was removed from the extract with a rotating evaporator.

Esterifications

Triglycerides were converted to methyl esters by refluxing 2 hr with 1% H_2SO_4 in methanol (w/v). After diluting the reaction mixture with an equal volume of water, esters were isolated by extraction with petroleum ether-ethyl ether (1:1) and subsequent removal of solvent with a rotating evaporator.

Reference Materials

The following reference materials were used: (a) 1,2-distearoyl-3-acetyl *sn*-glycerol prepared from egg yolk lecithin (4,5); (b) commercial distearin (Hormel Institute Austin, MN) acetylated to provide a mixture of acetodistearins; (c) cel-ATG isolated by countercurrent distribution of *C. orbiculatus* seed oil (6).

Chromatographic Methods

Thin Layer Chromatography (TLC): TLC, both analytical and preparative, was carried out on precoated silica gel F-254 plates (E. Merck, Darmstadt, Germany) with the solvent system petroleum ether-ethyl ether (2:1) for triglycerides, or petroleum ether-ethyl ether (3:2) for lipolysis products. In analytical TLC, components were visualized by charring with sulfuric acid-dichromate solution or by exposure to iodine vapor. In preparative TLC, bands were located by spraying with ethanolic dichlorofluorescein solution.

Gas Liquid Chromatography: GLC analyses of triglycerides and silylated lipolysis products were conducted with an F + M Model 810 instrument which has a flame ionization detector. A 3 ft x 1/8 in. stainless steel column packed with 3% OV-1 on Gas Chrom Q was used, temperature programmed 150-400 C at 6 C/min. Methyl esters were analyzed in a Hewlett-Packard Model 402 instrument with flame ionization detector. Two columns were operated isothermally at 180 C: (a) a 4 ft x 1/4 in. glass column packed with 5% Apiezon L on Chromosorb W and (b) a 10 ft x 1/4 in. glass column containing 5% Resoflex LAC-2R-446 on Chromosorb W. Methyl esters were identified by their equivalent chain length values.

Spectrometric Methods

A Perkin-Elmer model 700 spectrometer was used to determine IR spectra with 1% chloroform solutions.

NMR spectra were recorded with a Varian HA-100 instrument. Samples were dissolved in

[1]Presented at AOCS Meeting, New York, May 1977.

TABLE I

Fatty Acid Composition of *Polygala virgata* Seed Oil and Related Triglyceride Fractions (excluding acetic acid)[a]

Methyl ester	*Polygala virgata* seed oil	*P. virgata* acetotriglyceride (poly-ATG)	*Celastrus orbiculatus* acetotriglyceride[b] (cel-ATG)
14:0	5.9	6.4	0.2
16:0	22.1	25.8	16.4
18:0	7.0	9.5	1.6
18:1	43.5	44.5	5.0
18:2	9.9	6.3	33.5
18:3	0.7	0.3	42.9
20:1	3.5	5.2	---

[a]Upon hydrolysis, each ATG included in this table releases 1 mole of acetic acid per mole of triglyceride. Table I gives only the composition of the remainder of the acids liberated.

[b]The ATG used was a countercurrent fraction, not a composite of the entire ATG of *C. orbiculatus* seed oil (see ref. 6).

carbon tetrachloride with tetramethylsilane as an internal standard. For shift reagent studies, a carbon tetrachloride solution (0.5 g/ml) of deuterated tris(1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-4,6-octanedionato) europium (III) [$Eu(fod)_3 \cdot d_{27}$] was added to the sample in 10 μl increments to give the concentration required for optimum resolution of overlapping signals.

Gas chromatography-mass spectrometry (GC-MS) was carried out with a DuPont (CEC) 21-492-1 mass spectrometer essentially as described by Kleiman and Spencer (7). Samples were introduced into the mass spectrometer through a Bendix 2600 gas chromatograph and a stainless steel jet separator. The gas chromatograph was equipped with a 2 m x 2.0 mm glass column packed with 3% OV-1 on Gas Chrom Q. The temperature was programmed 4 C/min from 190 to 250 C. The inlet was at 230 C, the transfer line and jet separator at 250 C, and the mass spectrometer source was kept as 230 C. The filament current was 250 μA and the ionizing voltage, 70 eV.

The optical rotatory dispersion (ORD) spectrum was recorded with a Cary Model 60 spectropolarimeter. The sample was dissolved in hexane (7.2 g/100 ml).

Hydrolyses with Pancreatic Lipase

Hydrolysis of triglyceride samples was carried out with porcine pancreatic lipase (EC 3.1.1.3) (Calbiochem, Elk Grove Village, IL) by the procedure of Luddy et al. (8) as modified by Phillips and Smith (2). Reaction times of 30 min generally were required. In order to remove inorganic ions, the final aqueous phase in the workup was passed successively through columns of Dowex 50W-X4 (H^+ form) and AG1-X4 (OH^- form – Bio-Rad, Richmond, CA). The aqueous effluent then was taken to dryness with a rotating evaporator to isolate water-soluble lipolysis products. After fractionation of lipolysis products by preparative TLC, 1-mg samples were silylated and analyzed by GLC and GC-MS. The procedure of Tallent and Kleiman (9) was used for GLC, except that the silylating reagent was bis-(trimethylsilyl)-trifluoroacetamide (Regisil, Regis Chemical Co., Morton Gove, IL).

Hydrogenation

Hydrogenation of triglycerides was conducted in hexane solution at ambient temperature and pressure. Platinum oxide catalyst was used and was removed by filtration after completion of the reaction.

RESULTS AND DISCUSSION

According to GLC analyses of conventional methyl ester preparations, the fatty acid composition of *Polygala* seed oil is quite ordinary (Table I). The IR spectrum was generally typical of triglycerides except for a prominent peak at 1370 cm^{-1}, apparently due to the presence of acetate groupings. Glyceride analysis by GLC revealed that about 68% of the oil was more volatile than ordinary triglycerides with three long chain acyl groups.

TLC indicated that the oil contains relatively little triglyceride with the usual chromatographic mobility, but is comprised mainly of a constituent of a lower R_f suggesting an ATG. However, this substance has a distinctly higher R_f than cel-ATG, which is known to have its acetate group in position 3 of *sn*-glycerol (Fig. 1) (4,6). As displayed in Fig. 1, *P. virgata* seed oil has little resemblance to that of the

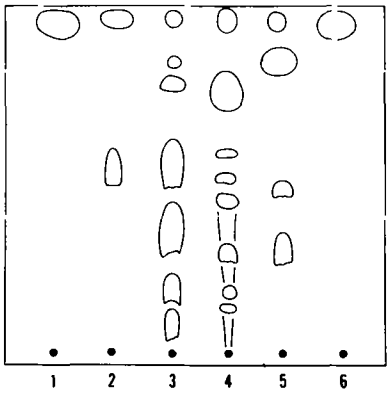

FIG. 1. Thin layer chromatogram of seed oils from: 1,6, soybean; 2, *Monnina emarginata;* 3, *Securidaca longipedunculata;* 4, *Celastrus orbiculatus;* 5, *Polygala virgata.* Adsorbent = silica gel F-254; solvent = petroleum ether-ethyl ether (2:1).

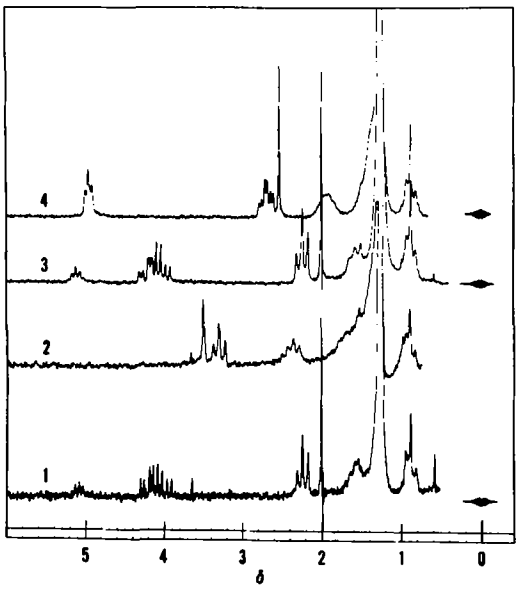

FIG. 2. Nuclear magnetic resonance spectra illustrating determination of ATG structures by use of a shift reagent. 1 = Hydrogenated ATG from *Polygala virgata* seed oil in carbon tetrachloride solution (80 mg/ml); 2 = sample 1 after addition of shift reagent to make the solution 0.012 M $Eu(fod)_3$-d_{27}; 3 = hydrogenated ATG from egg lecithin in carbon tetrachloride (76 mg/ml); 4 = sample 3 after addition of shift reagent to make the solution 0.02 M $Eu(fod)_3$-d_{27}.

previously investigated *Monnina emarginata.* The seed oil of a third polygalaceous species, *Securidaca longipedunculata,* shows greater complexity than either of the other two and combines some features of both.

P. virgata seed oil (91.7 mg) was separated by preparative TLC into the following compo-

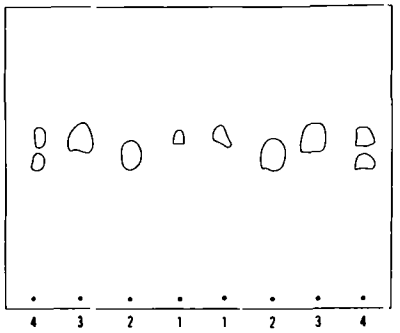

FIG. 3. Thin layer chromatogram of: 1, hydrogenated ATG from *Polygala virgata* seed oil; 2, ATG from hydrogenated egg lecithin; 3, ATG from *P. virgata* before hydrogenation; 4, mixture of 1(3)-acetodistearin (lower spot) and 2-acetodistearin (upper spot). Solvent and adsorbent: see Figure 1.

nents (in order of descending R_f): I, 14.4 mg; II, 68.3 mg; III, 4.1 mg; IV, 8.0 mg. The major component, fraction II, subsequently identified as poly-ATG, was hydrogenated to give poly-ATGH. Presence of acetate in groups in poly-ATGH was confirmed by NMR (Fig. 2), which showed a strong singlet resonance at δ2.0, and by IR, which revealed enhancement of the absorption at 1370 cm^{-1}. GLC analysis of poly-ATGH showed the following glyceride composition: $C_2C_{14}C_{14}$, 3.9%; $C_2C_{14}C_{16}$, 1.9%; $C_2C_{16}C_{16}$, 10.5%; $C_2C_{16}C_{18}$, 3.9%; $C_2C_{18}C_{18}$, 22.6%; $C_2C_{18}C_{20}$, 3.3%. TLC examination of poly-ATGH led to the inference that it is a 2-acetotriglyceride, since its R_f coincides with that of 2-acetodistearin, the upper spot from the mixture of acetodistearins (Fig. 3). Conversely, the known 3-acetotriglyceride in Figure 3 (egg-ATGH) corresponds in R_f to the lower spot from this mixture.

Poly-ATGH is optically inactive within experimental error as demonstrated by spectropolarimetric measurements throughout the accessible range (340-600 nm). Accordingly, poly-ATGH should have its acetate group in position 2 of *sn*-glycerol since this is the only one of the three possible isomers (Fig. 4) that would be optically inactive. Previous work (4,10,11) has shown that 3-acetyl dipalmitins or distearins have measurable, though slight, optical rotations with plain, negative ORD curves. The enantiomeric 1-acetyl triglycerides would be expected to show plain, positive ORD curves of the same magnitude. In contrast, the 2-acetyl isomer has a plane of symmetry and should be optically inactive (10).

The acetate group of poly-ATGH can be definitely allocated to position 2 through evidence provided by NMR studies with a shift reagent. Previous research has shown that

lanthanide shift reagents can be used to distinguish aliphatic methylene groups whose NMR signals normally overlap. In triglycerides, even α- and β-methylene groups of similar fatty acyl groups may be shifted to a different degree under the influence of a shift reagent, depending on the nature of the acyl groups and where they are attached to the glycerol moiety (12-16).

The NMR spectrum of poly-ATGH showed a triplet at δ2.27 (J = 7 Hz) (Fig. 2) as expected for the α-methylene group of a saturated long chain acyl group. After addition of an appropriate concentration of shift reagent (see legend, Fig. 2), this triplet was shifted downfield to δ3.30, but remained a discrete triplet. The acetate singlet at δ2.0 is displaced downfield by the shift reagent more than the methylene triplet, so that the relative position of the two is reversed. Without the influence of a shift reagent, the corresponding NMR spectrum of egg-ATGH was very similar to that of poly-ATGH. However, upon addition of $Eu(fod)_3 \cdot d_{27}$, the downfield shift of the δ2.27 triplet was accompanied by its resolution into two clearly discernible sets of signals separated by ca. 3 Hz (Fig. 2). These observations are in accord with prior evidence that egg-ATGH has an unsymmetrical structure with its acetate group at position 3 (Fig. 4). Accordingly, poly-ATGH must have the symmetrical 2-acetate structure with both stearoyl or palmitoyl groups in identical magnetic environments.

poly-ATGH was treated with pancreatic lipase (EC 3.1.1.3) because of this enzyme's known specificity for positions 1 and 3 of triglycerides. We anticipated that glycerol-2-acetate would be the ultimate product of the reaction. However, pancreatic lipolysis of poly-ATGH gave inconclusive results. Since monoacetins are water soluble, we modified the standard lipolysis procedure in order to isolate water-soluble products. However, we were unable to detect any monoacetin after lipolysis of poly-ATGH, although glycerol was detected in the final aqueous phase. This observation possibly indicates that 2-monoacetin is hydrolyzed rapidly by some mechanism in the aqueous phase and, consequently, does not accumulate (17,18). Further investigation is required to determine whether isomeric monoacetodiglycerides can be distinguished and identified by MS, since GC-MS of our lipolysis products gave inconclusive results.

Acetotriglycerides are quite restricted in their natural occurrence. Known examples from plant sources include seed oils of several genera in the family Celastraceae (4,6,10) and of the genus *Impatiens* in the Balsiminaceae (10,19).

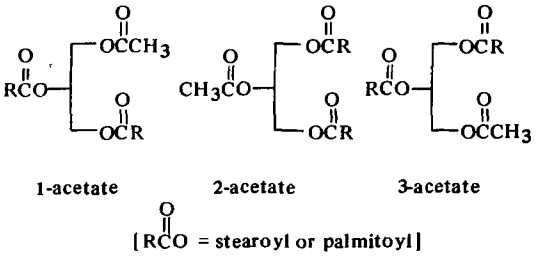

FIG. 4. The three stereoisomeric forms of acetotriglycerides.

The most recently discovered source of ATG, also the first found in the animal kingdom, is the lipid of the scale insect *Icerya purchasi* (11). ATG from all three of these sources are optically active with the acetate group exclusively in position 3 of *sn*-glycerol (4,10,11,19). Thus, poly-ATG is the first example of a new class of ATG stereospecifically synthesized with the acetate group in position 2.

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Fatty Acid Composition of Selected Organs of Gerbils Maintained on a Fat-deficient or Fat-supplemented Diet

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ABSTRACT

The fatty acid composition of liver, heart, and testes was determined in gerbils maintained on a fat-deficient or fat-supplemented diet since the age of twenty-eight days and in gerbil pups, the mothers of which were placed on the respective diets on the day of delivery. Pups born to these mothers were killed at 11 to 19 days at which time increased concentrations of 16:1, 18:1, and 20:3ω9 and decreased concentrations of 18:2 and 20:4 (and 22:5ω6 in testes) were apparent in organs of fat-deficient compared to fat-supplemented gerbils. Similar but more marked changes occurred in organs of gerbils placed on the fat-deficient diet at twenty-eight days of age and examined at intervals of time up to two months later. In these animals, minimal changes were seen also in fatty acids of the brain. The concentration of 22:6ω3 was resistant to change with the fat-deficient diet. Deficient gerbils had hair loss, decreased quantities of spermatids and spermatozoa in the testis, and most but not all had decreased body weight compared to the fat-supplemented controls. Extremely high concentrations of oleic acid were present in carcass fatty acids of deficient compared to supplemented gerbils, indicating an extremely dynamic fatty acid metabolism in these animals.

INTRODUCTION

Although investigations of essential fatty acid deficiency have been made in many species of animals, we are aware of only one reference to the use of the mongolian gerbil (*Meriones unguiculatus*) for such studies. This is a statement to the effect that feeding the gerbil a fat-free diet produced no overt symptoms of essential fatty acid (EFA) deficiency (1).

The gerbil, which can live almost entirely without drinking water and which produces very little urine, was found to develop high serum and hepatic cholesterol levels when fed low levels of fat, but no gross atherosclerotic changes could be found in the aorta (2). Numerous other studies of cholesterol metabolism in the gerbil have been reported (3,4).

Albers and Gordon reported that arachidonic acid was virtually absent from the cholesteryl ester fraction of gerbil plasma (5). This led Gordon and Mead to study the conversion of linoleic to arachidonic acid in the gerbil. They observed that linoleic acid-1-^{14}C administered orally as the methyl ester was converted to arachidonic acid (6).

Because of the lack of published data on the response of this unique animal to a fat-deficient diet, we have studied the changes which occur

TABLE 1

Fatty Acid Composition of Selected Organs of 24-Day-Old Gerbils Maintained on a Fat-deficient or Fat-supplemented Diet Since Birth

Fatty acid[a]	% of total fatty acids					
	Liver		Heart		Testes	
	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient
16:0	21.4	26.4	23.5	12.1	32.3	23.4
16:1	0.2	0.8	0.6	1.0	1.0	1.9
18:0	15.6	18.4	20.2	21.1	12.1	18.9
18:1	19.5	29.6	14.2	21.7	27.0	32.1
18:2	25.3	1.6	20.2	5.4	10.2	0.8
20:3ω9	ND[b]	7.2	0.1	10.2	ND	9.1
20:4	13.1	7.2	15.3	12.1	11.5	6.0
22:5	ND	0.2	0.2	1.0	6.0	0.8
22:6	2.4	5.2	13.5	12.5	0.7	0.8

[a]Number of carbons:number of double bonds.

[b]ND = none detected.

TABLE II

Body and Organ Weights (in grams) of Gerbils Maintained on a Fat-deficient or Fat-supplemented Diet from the Age of 28 Days

Males									
Days on diet	11		26		42		59		
Age (days)	39		54		70		87		
Weight of	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient	Supplemented		Deficient
Body	51	44	58	35	54	63	65		68
Liver	2.23	1.89	2.32	1.67	2.11	3.38	2.55		3.51
Heart	0.26	0.19	0.25	0.19	0.26	0.33	0.26		0.25
Brain	0.72	0.69	0.95	0.91	0.94	1.00	0.93		0.96
Testes	0.58	0.42	0.81	0.68	0.95	0.83	1.10		0.90
Females									
Days on diet	13		28		43		55		
Age (days)	39		56		71		83		
Weight of	Supplemented	Deficient	Supplemented	Deficient	Supplemented	Deficient	Supplemented		Deficient
Body	44	36	53	42	59	56	59		43
Liver	1.91	1.63	2.38	2.33	2.42	2.61	2.29		1.70
Heart	0.21	0.16	0.21	0.22	0.23	0.25	0.22		0.20
Brain	0.69	0.68	0.94	0.90	0.98	0.90	0.93		0.87

in the fatty acid composition of selected organs of gerbils placed on a fat-deficient diet. Only limited observations were made on clinical signs of essential fatty acid deficiency in these animals.

MATERIALS AND METHODS

Gerbils were obtained from Tumblebrook Farm, Inc., West Brookfield, MA. Our initial analyses were made on pups born to mothers maintained from the day of delivery on a purified diet containing 10% corn oil (fat-supplemented) or containing no fat (fat-deficient). The diet is one that has been used in our laboratory for the rat (7), and it was supplemented only with lettuce given ad libitum to all gerbils. Between the time of arrival and the day of delivery, the gerbils were maintained on Purina Laboratory Chow (supplemented daily with lettuce). One deficient and one supplemented pup were killed at 11, 19, and 24 days (Experiment 1).

The gerbils of Experiment 2 were received from the suppliers when they were 28-days-old and they were placed on the fat-deficient or fat-supplemented diet at that time. A male deficient and a supplemented animal were killed after 11, 26, 42, and 59 days on the diet and a female deficient and a supplemented animal were killed after 13, 28, 43, and 55 days on the diet.

The gerbils were killed by decapitation and organs selected for study were removed immediately and placed in alcoholic KOH containing hydroquinone. Methods for isolation of fatty acids and quantitative analysis of the methyl esters by gas chromatography have been reported previously (8,9). Gas chromatography was done on all samples before and after hydrogenation in order to assure identity of the fatty acids. Hydrogenation was done by the method of Farquhar et al. (10).

The total fatty acid content of lettuce was determined by titration after extraction of the acidified KOH hydrolysate of a weighed quantity of lettuce. The fatty acid composition was determined by gas chromatography of the methyl esters.

RESULTS

The body weights of the gerbil pups of Experiment 1 were (in grams) 5.8, 10.1, and 12.0 for the 11_s, 19-, and 24-day-old supplemented, respectively, and 3.6, 3.6, and 5.9 for the 11-, 19-, and 24-day-old deficient animals, respectively. In Table I is a summary of the main fatty acid composition of liver, heart, and testes of the 24-day-old gerbils. The fatty acid compo-

TABLE III

Fatty Acid Composition of Livers of Gerbils
Maintained on a Fat-deficient or Fat-supplemented Diet from the Age of 28 Days

	% of total fatty acids[a]			
	Males		Females	
Fatty acid	Supplemented	Deficient	Supplemented	Deficient
16:0	17.9 ± 1.5	18.4 ± 0.7	17.4 ± 0.7	17.6 ± 1.7
16:1	0.6 ± 0.27	5.3 ± 0.2	1.7 ± 0.5	4.0 ± 1.2
18:0	18.8 ± 1.0	11.9 ± 0.8	16.8 ± 1.2	12.8 ± 0.9
18:1	16.1 ± 1.6	50.5 ± 2.4	26.5 ± 4.6	50.7 ± 1.3
18:2	27.5 ± 1.5	2.0 ± 0.9	22.5 ± 1.6	1.9 ± 0.5
20:3ω9	0.1 ± 0.05	7.6 ± 0.6	0.08 ± 0.05	7.2 ± 0.8
20:3ω6	1.6 ± 0.3	0.15 ± 0.05	2.2 ± 0.4	0.3 ± 0.08
20:4	15.4 ± 1.9	2.6 ± 0.8	10.7 ± 1.1	3.5 ± 0.7
22:6	1.7 ± 0.3	0.55 ± 0.07	1.8 ± 0.2	1.4 ± 0.1

[a]Average ± SE for all time periods.

TABLE IV

Fatty Acid Composition of Hearts of Gerbils Maintained on a
Fat-deficient or Fat-supplemented Diet from the Age of 28 Days

	% of total fatty acids[a]			
	Males		Females	
Fatty acid	Supplemented	Deficient	Supplemented	Deficient
16:0	15.3 ± 0.8	15.1 ± 0.6	15.3 ± 0.8	15.1 ± 1.2
16:1	0.5 ± 0.2	4.1 ± 0.1	0.5 ± 0.2	3.8 ± 0.8
18:0	17.6 ± 1.0	16.7 ± 0.9	17.9 ± 1.6	17.4 ± 0.9
18:1	17.0 ± 1.4	33.5 ± 2.2	15.6 ± 1.8	31.8 ± 1.6
18:2	33.4 ± 2.2	9.6 ± 2.3	34.2 ± 1.4	10.7 ± 2.9
20:3ω9	<0.1	7.5 ± 1.9	<0.1	7.2 ± 2.2
20:3ω6	0.2 ± 0.1	0.2 ± 0.06	0.3 ± 0.1	0.4 ± 0.07
20:4	10.8 ± 1.9	9.1 ± 0.4	11.0 ± 1.7	8.9 ± 0.7
22:6	4.9 ± 1.1	4.3 ± 1.1	4.6 ± 1.3	4.7 ± 1.2

[a]Average ± SE for all time periods.

sition of the 11- and 19-day-old animals generally was not far different than that of the 24-day-old and the main differences are pointed out below. Higher values for 16:1, 18:1, and 20:3ω9 and lower values for 18:2 and 20:4 were apparent in organs of the fat-deficient compared to the supplemented gerbils at the three ages. In testes of the deficient animals, the concentration of 22:5ω6 was extremely low compared to the supplemented ones. In the three organs studied, the concentration of 20:3ω9 increased three to fivefold from the 11-day value to the 24-day value. Although the concentration of 20:4 in liver and testis of deficient was lower than that in supplemented gerbils, the concentration in the heart was only slightly lower in the deficient group (12.1, 13.9, 12.1 for deficient and 16.5, 17.9, 15.3 for supplemented gerbils). The concentration of 22:6 was as great or greater in testis and liver of deficient than in corresponding organs of supplemented gerbils. After removal of the liver, heart, and testis, the remaining organs and carcass were processed as one sample. The same types of differences in fatty acid composition between the two groups were observed as had been observed in the organs studied separately.

The body and organ weights of the gerbils in Experiment 2 are given in Table II. In five of the eight pairs, the body weight of the deficient was lower than that of the corresponding supplemented animal. Suggestive differences in organ weights between supplemented and deficient animals in the youngest age bracket had largely disappeared in the two older age groups. The fatty acid composition of liver, heart, brain, and testis is given in Tables III, IV, V, and VI.

In the liver (Table III), increased concentrations of 16:1, 18:1, and 20:3ω9 and decreased concentrations of 18:0, 18:2, 20:3ω6, 20:4, and 22:6 were seen in male and female deficient compared to supplemented animals. In the heart (Table IV), increased concentrations

TABLE V

Fatty Acid Composition of Brains of Gerbils Maintained on a Fat-deficient or Fat-supplemented Diet from the Age of 28 Days

	% of total fatty acids[a]			
	Males		Females	
Fatty acid	Supplemented	Deficient	Supplemented	Deficient
16:0	26.0 ± 1.2	25.3 ± 0.5	25.6 ± 0.9	23.8 ± 1.0
16:1	0.3 ± 0.1	0.6 ± 0.07	0.3 ± 0.07	0.3 ± 0.04
18:0	22.3 ± 0.7	22.4 ± 0.3	23.1 ± 0.6	21.7 ± 0.2
18:1	23.7 ± 0.7	25.9 ± 0.7	22.7 ± 1.2	26.1 ± 0.5
18:2	0.9 ± 0.1	0.1 ± 0.06	1.2 ± 0.2	0.2 ± 0.1
20:3ω9	ND[b]	1.6 ± 0.4	ND	1.3 ± 0.5
20:4	10.3 ± 0.1	8.3 ± 0.8	10.0 ± 0.3	9.0 ± 0.4
22:4	1.7 ± 0.3	1.6 ± 0.2	2.4 ± 0.3	1.5 ± 0.2
22:6	12.8 ± 0.5	12.1 ± 0.5	12.6 ± 0.5	14.1 ± 0.4

[a]Average ±SE for all time periods.
[b]ND = none detected.

TABLE VI

Fatty Acid Composition of Testes of Gerbils Maintained on a Fat-deficient or Fat-supplemented Diet from the Age of 28 Days

	% of total fatty acids[a]	
Fatty acid	Supplemented	Deficient
16:0	37.5 ± 1.9	39.9 ± 1.6
16:1	0.6 ± 0.1	2.5 ± 0.3
18:0	4.7 ± 0.4	5.5 ± 0.4
18:1	16.6 ± 1.5	30.9 ± 3.0
18:2	12.6 ± 1.3	1.2 ± 0.7
20:3ω9	ND[b]	5.9 ± 1.5
20:4	13.2 ± 0.5	4.5 ± 0.5
22:5ω6	14.0 ± 0.5	5.9 ± 0.4
22:6	0.5 ± 0.2	1.0 ± 0.3

[a]Average ± SE for all time periods.
[b]ND = none detected.

of 16:1, 18:1, and 20:3ω9 and decreased concentrations of 18:2 were found in the male and female deficient gerbils. Values for both 20:4 and 22:6 were similar for the two groups (contrast with liver). The concentration of 22:6 in hearts of the youngest age group was higher than in the oldest animals (both males and females, deficient and supplemented). Minimal changes were seen in brain (table V). A definite increase in the deficient group was seen only in 20:3ω9 and a definite decrease was seen only in 18:2. It is probable that the amounts of 16:1 and 18:1 were increasing in the deficient gerbils. In testes (table VI), there were increases in 16:1, 18:1, and 20:3ω9 and decreases in 18:2, 20:4, and 22:5 in the deficient compared to the supplemented gerbils. An additional deficient male and an additional deficient female, for which there were no controls, were killed when they had been on the diet for 69 days. Values for fatty acids of all organs studied were similar to those obtained for the oldest gerbils reported in Tables II-VI.

Several pups from other litters were also used in studying the effect of the fat-deficient or fat-supplemented diet on fatty acid composition. The appearance of a significant quantity of 20:3ω9 was noted even in a 7-day-old pup, the mother of which had been on a fat-deficient diet since delivery. In liver 1.1% and in total body 0.1% of the total fatty acids was due to 20:3ω9 in this pup. In a 14-day-old pup from the same litter, these values were 1.9 and 0.3%, respectively. Another pair of pups was kept on a fat-deficient or fat-supplemented diet for 5 months after having been on a fat-supplemented diet for the first month after birth. The deficient animal was smaller (46 g vs. 58 g) and had smaller testes (0.13 g vs. 0.69 g) than the fat-supplemented one. The fatty acid composition of liver, testis, and heart showed the same types of changes noted in the gerbils of Tables II-VI but of a greater degree. In the carcass, 73.2% of the total fatty acids in the deficient gerbil were 18:1 compared to 36.3 in the fat-supplemented one. The mothers of these pups were killed at this time. The fat-deficient gerbil was smaller than the supplemented gerbil (64 g vs. 84 g) and had large amounts of 20:3ω9 in liver (14.9% of total fatty acids) and in heart (10.8% of total fatty acids). In the carcass of the deficient gerbil, 62.3% of the total fatty acids were 18:1 compared to 40.6 in the supplemetned gerbil.

Histological examination of the testes of gerbils in Experiment 2 revealed some differences between the supplemented and the deficient gerbils. Generally, the quantities of spermatids and spermatoza in testes of the deficient

animals were significantly less than in those of supplemented animals. However, there were no signs of degeneration in testes of the deficient gerbils at this time.

Gerbils that had been maintained on the fat-deficient diet from age 28 days suffered loss of hair (mostly on the back near the tail) by the end of 2 to 3 months on the diet. Two males that had been on the fat-deficient diet for 3 months and which had developed hair loss had stopped gaining weight and generally looked very sick, were switched to the diet containing 10% corn oil. Within 1 month, during which time weight gain commenced, the lost hair had been restored and within 2 months the body weight was comparable to that of the fat-supplemented pair, and the animals appeared completely healthy.

The total fatty acid content of the lettuce was 0.77 mg/g and the 18-carbon polyenoic acids present were (as % of total fatty acids): linoleic – 31.8 and α-linolenic – 37.7. An estimate of the probable maximal amount of these polyenes ingested daily by each gerbil based on its consumption of lettuce is (in mg): linoleic – 0.3 and α-linolenic – 0.4.

DISCUSSION

The observations made and the data obtained in these studies indicate that gerbils respond to a fat-deficient diet in at least some of the same ways as do other species. Although only limited clinical observations were made (body weight, loss of hair, general appearance), these support the chemical evidence of essential fatty acid deficiency. The chemical evidence consisted of the appearance of 20:3ω9, increased concentrations in the organs studied of 16:1 and 18:1, and decreased concentrations of 18:2 and 20:4 and, in the case of the testes, of 22:5ω6. That fat deficiency can develop readily in gerbils is indicated by the finding of a significant quantity of 20:3ω9 in the liver and carcass of a 7-day-old pup, the mother of which was placed on the fat-deficient diet on the day of delivery. That the absence of fat in the diet was responsible for the effects seen was shown by the recovery made by deficient animals that were given the corn oil diet after developing the deficiency.

It should be noted that although the lettuce supplement provided some essential fatty acids, the total amount was too small to prevent essential fatty acid deficiency from developing.

The very dynamic fatty acid metabolism occurring in the gerbil was demonstrated by the markedly high concentration of oleic acid in the deficient compared to the supplemented animal. In the carcass lipid of one deficient gerbil, 73% of the fatty acid content was oleic acid, compared to 36% for the supplemented gerbil.

On the other hand, the resistance of the polyene 22:6ω3 to a change in concentration due to fat deficiency was demonstrated by the values seen in these studies, particularly in the heart. It is also of interest that the concentration of 22:6ω3 was highest in the hearts of the youngest age groups compared to the oldest (both male and female, deficient and supplemented).

The higher standard error values for certain fatty acids (18:2, 20:3ω9, 18:1) in Tables III-VI are an indication of differences among the animals of various ages due to the developing deficiency.

The low values for 22:5ω6 seen in testes of deficient animals in the older age groups (Table VI) correlate with the decreased amounts of spermatids and spermatoza seen histologically in these animals. It is though that in testes of rats this polyenoic fatty acid is specifically associated with spermatids and spermatoza (11).

It should be noted that the complete dietary requirements of the gerbil are not known. The animals used in this study were maintained on a diet that has prove adequate for the rat. Gerbils have been kept on this diet for as long as 9 to 10 months in apparently good health and with the ability to reproduce.

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Modification of the Fatty Acid Composition of L1210 Murine Leukemia Cells

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ABSTRACT

We have compared the effect of diets containing 16% sunflower seed oil (polyunsaturated fat-rich) or 16% coconut oil (saturated fat-rich) fed for 3-7 weeks on the composition of L1210 murine leukemia cells which were transplanted into the peritoneal cavity during the final week of feeding. The L1210 phospholipids of mice fed the sunflower oil diet contained 43% polyenoic fatty acids and an average of 1.5 double bonds per fatty acid molecule as compared to only 25% polyenoic fatty acids and 1.2 double bonds in the coconut oil group. In contrast, the cells from the sunflower oil group contained only 13% monoenoic fatty acids as compared to 33% in those from the coconut oil group. When compared to phospholipids of tumors from mice who were fed a commercial mouse chow, cells grown on sunflower oil had an 18% increase in polyenoic fatty acids and those grown on coconut oil a 31% decrease. The greatest changes occurred in the proportion of oleate and linoleate. There was only a small difference in the percentage of saturated fatty acids and in the mean fatty acid chain length among the tumor cells from animals on the experimental diets. The changes in the fatty acid composition of the L1210 cell neutral lipids and the lipids of the ascites fluid were similar to those observed in the phospholipids. A majority of the changes had occurred after 5 weeks of feeding the special diet. These results indicated that the fatty acid saturation of tumor cell phospholipids can be altered appreciably. The changes in fatty acid composition were not associated with any change in the sterol/phospholipid ratio of the cells. Therefore, our results suggest that it may be possible to alter the physical properties and function of a tumor cell membrane by dietary modification of its phospholipid composition.

INTRODUCTION

Recent studies indicate that L1210 cells freshly obtained from host mice are able to take up large quantities of fatty acids and incorporate them into cell lipids during short term in vitro incubations (1). Experiments with radioactive fatty acids indicate that they are taken up and incorporated into tumor cell lipids for the most part without elongation or desaturation. This suggests that it may be possible to alter the fatty acid composition of the L1210 cell lipids by varying the type of fatty acids supplied to the tumor cell from external sources. Such diet-induced modifications of the fatty acid composition of Ehrlich ascites tumor cells (2) and rat hepatoma (3) have been produced. Therefore, we have investigated the effect of lipids of various degrees of saturation contained in the diet of host mice on the composition of the L1210 lipids. We chose the L1210 murine leukemia for study since its sensitivity to antineoplastic agents is similar to many human tumors (4). Since the neoplastic process is associated with abnormalities in the physical structure and function of cell membranes (5), we focused most of our attention initially on the phospholipids of the L1210 cell.

MATERIALS AND METHODS

Male DBA/2j mice, initially weighing 16-18 g (Jackson Laboratories, Bar Harbor, ME) were fed basal semisynthetic diets (Teklad Test Diets, Madison, WI) supplemented with either 16% coconut oil or 16% sunflower oil (Ruger Chemical Company, Inc., Hillside, NJ) for 2, 4, or 6 weeks prior to intraperitoneal injection of 1.5×10^5 L1210 tumor cells. The source and method of transplantation of the tumor cells have been described (1). The diet was continued for an additional week after transplantation while the tumor grew. The sunflower oil and melted coconut oil were weighed and mixed with the powdered basal semisynthetic diet using a blender. The coconut oil diet contained 93% saturated fatty acids while the sunflower oil diet consisted principally of unsaturated fatty acids, primarily linoleic acid (71%). The contents and fatty acid composition of the diets have been described (6). Other animals were fed standard mouse chow (Teklad Mills, Winfield, IA) containing 4.48% fat for 8 weeks prior to injection of tumor cells (1). Data on this chow were included in order to provide

TABLE I

Effect of Diet on Fatty Acid Percentage Composition of the L1210 Cell Phospholipids

Fatty acid	Percentage of fatty acids[a]						
	Sunflower seed oil diet			Coconut oil diet			
	Weeks			Weeks			Commercial mouse chow
	3	5[c]	7	3	5[c]	7	
<14.0	0.1 ± <0.1	1.8 ± 0.3	0.5 ± 0.2	0.2 ± 0.1	2.1 ± 0.6	1.3 ± 0.5	1.3 ± 0.3
14:0	0.8 + 0.1	1.8 ± 0.3	1.0 ± 0.1	1.9 ± 0.3	3.9 ± 0.5	3.1 ± 0.3	1.6 ± 0.2
16:0	16.2 ± 0.6	16.9 ± 0.6	15.7 ± 0.3	15.8 ± 0.5	16.9 ± 0.4	15.1 ± 0.3	18.3 ± 0.6
16:1	1.7 ± 0.1	1.6 ± 0.2	1.4 ± 0.1	3.7 ± 0.1	4.4 ± 0.4	4.8 ± 0.2	2.4 ± 0.1
18:0	22.0 ± 1.4	19.5 ± 0.7	19.5 ± 1.1	20.5 ± 0.7	16.4 ± 1.0	17.6 ± 0.3	18.7 ± 0.3
18:1	13.2 ± 0.4	11.1 ± 0.4	10.6 ± 0.4	24.5 ± 0.5	27.4 ± 0.9	28.3 ± 0.7	18.1 ± 0.3
18:2	23.4 ± 0.6	23.5 ± 1.0	27.7 ± 2.3	17.0 ± 0.2	11.7 ± 0.8	10.7 ± 0.6	18.3 ± 0.4
20:4	10.8 ± 0.7	10.7 ± 0.7	10.1 ± 0.8	9.0 ± 0.2	6.5 ± 0.7	7.5 ± 0.5	9.0 ± 0.2
22:0	2.7 ± 0.1	2.6 ± 0.1	3.2 ± 0.2	0.3 ± 0.1	0.4 ± 0.1	0.4 ± <0.1	1.4 ± 0.1
22:4	2.7 ± 0.1	2.6 ± 0.2	2.7 ± 0.1	0.6 ± 0.2	0.4 ± 0.2	0.6 ± 0.1	1.7 ± 0.1
22:5	1.7 ± 0.4	2.5 ± 0.2	1.9 ± 0.2	2.0 ± 0.3	3.9 ± 1.6	2.4 ± 0.4	2.5 ± 0.2
22:6	3.2 ± 0.3	3.3 ± 0.1	3.2 ± 0.3	2.0 ± 0.1	1.3 ± 0.2	1.7 ± 0.3	3.7 ± 0.1
Others[b]	1.5 ± 0.2	2.1 ± 0.5	2.5 ± 0.9	2.5 ± 0.1	4.7 ± 1.0	6.5 ± 1.5	3.0 ± 0.4

[a]Values shown are the percentage composition and are the mean and SEM of determinations on individual samples from 4-7 mice. The time on the experimental diets includes a 2, 4, or 6 week period of feeding prior to injection of tumor plus an additional week of feeding the experimental diet during growth of the tumor.

[b]Includes small amounts of 18:3, 20:1, 20:3, 20:5, 22:1, and unidentified fatty acids.

[c]At 5 weeks, the percentage of the following fatty acids in the cells from the animals fed the sunflower oil diet were statistically different than the corresponding time period of cells from the animals fed the coconut oil diet: 16:1, 18:1, 18:2, 22:0, 22:4, 22:6 – $p<0.001$; 14:0, 20:4 – $p<0.01$; 18:0 – $p<0.05$.

information on the L1210 tumor as it is commonly carried in most laboratories and to provide data regarding the composition of the tumor cells prior to the experimental diets (week 0). Initially there were seven mice on each diet for each time point. There was no difference in rate of food consumption or weight of the animals among the diets.

Seven days after intraperitoneal injection of 1.5×10^5 L1210 cells, the mice were sacrificed by cervical dislocation and the cells were harvested, washed, and counted in duplicate as described previously (1). The washed cells and ascites fluids were extracted using $CHCl_3/CH_3OH$ (2:1, v/v) (7), and aliquots of the washed chloroform extract were dried under nitrogen and redissolved in hexane. Using this procedure, 94% of the phospholipids in the chloroform extract are transferred to the column. The lipid extracts were separated into neutral lipid and phospholipid fractions on 3 g heat-activated (100 C for 1 hr) silicic acid columns (7 x 31 mm) (Unisil, 100-200 mesh; Clarkson Chemical Company, Williamsport, PA) (8). Sequential elution with hexane (90 ml), chloroform (150 ml), acetone to remove glycolipids (90 ml), and methanol (90 ml) was carried out. The hexane fraction contained only a trace of triglycerides and fatty acids. The composition and purity of the chloroform (neutral lipids – tri-, mono-, and diglycerides, fatty acids and sterols) and methanol (phospholipid) eluates was confirmed by one-dimensional thin layer chromatography on Silica Gel G using a solvent system of hexane-diethyl-ether-methanol-acetic acid, 180:40:4:6.

The phospholipid and neutral lipid fractions from individual mice were saponified for 2 hr at 70 C in 6.7% KOH in 80% ethanol (9). The nonsaponifiable lipids were removed by extraction into hexane and the remaining suspension was acidified. The fatty acids were then extracted into hexane, and they were methylated for 10 min at 95 C with 14% BF_3-CH_3OH (10). A Hewlett-Packard 5710A Gas Liquid Chromatograph having a 6 ft by 0.25 in. glass column packed with Silar 10C on Gas Chrom Q (100-200 mesh) (11) was used to separate the individual fatty acid methyl esters. Peak areas were quantitated with a Hewlett-Packard Integrator 3380A. Peaks were identified by comparison of retention times to those of standards obtained from Applied Science Laboratories or Supelco, Inc. Additional aliquots of the cell lipid extracts were used to measure the phospholipid (12), sterol (13), and acylglycerol (13) content. Statistical analysis was done using the *t*-test.

TABLE II

Effect of Diet on Fatty Acid Percentage Composition of Neutral Lipids of L1210 Cells

Fatty acid	Percentage of fatty acid[a]		
	Sunflower seed oil diet[b]	Coconut oil diet[b]	Commercial mouse chow
<14:0	0.1 ± <0.1	2.2 ± 0.4	0.1 ± <0.1
14:0	1.4 ± 0.1	6.3 ± 0.3	1.6 ± 0.2
16:0	18.3 ± 0.1	22.7 ± 0.3	21.2 ± 0.5
16:1	3.8 ± 0.2	7.4 ± 0.4	5.0 ± 0.3
18:0	9.7 ± 1.3	10.6 ± 1.3	11.3 ± 0.1
18:1	21.3 ± 0.9	35.3 ± 0.5	29.9 ± 0.6
18:2	34.3 ± 2.2	9.4 ± 0.5	19.0 ± 0.7
20:1[c]	1.2 ± 0.4	2.2 ± 0.1	3.8 ± 0.7
20:4	2.8 ± 0.6	1.0 ± 0.2	2.2 ± 0.1
22:4	1.1 ± 0.2	0.2 ± <0.1	0.8 ± 0.1
22:5	0.8 ± 0.2	0.3 ± <0.1	1.1 ± <0.1
22:6	0.9 ± 0.2	0.3 ± <0.1	1.4 ± 0.1
Others[d]	4.3 ± 1.1	2.1 ± 0.4	2.6 ± 0.4

[a] Values shown are the percentage composition and are the mean and SEM of single determinations on individual samples from 6-7 mice. The values are those after 5 weeks of feeding.

[b] The percentage of the following fatty acids in cells from sunflower seed and coconut oil diet animals were statistically different: <14:0, 14:0, 16:0, 16:1, 18:1, 18:2 – $p<0.001$; 22:6, 22:4 – $p<0.01$; 20:1, 20:4, 22:5 – $p<0.05$.

[c] Includes 18:3 and 20:0.

[d] Includes small amounts of 20:2, 20:3, 20:5, 22:0, 22:1, 24:1, and unidentified fatty acids.

TABLE III

Differences in the Fatty Acid Composition of L1210 Cell Lipids

	Sunflower seed oil diet[a]		Coconut oil diet[a]		Commercial mouse chow	
	Phospholipids	Neutral lipids	Phospholipids	Neutral lipids	Phospholipids	Neutral lipids
Percent saturated	40.7 ± 0.2[b]	29.4 ± 0.6	37.6 ±<0.1	40.0 ± 0.6	40.0 ± 0.3	34.1 ± 0.2
Percent monoenoic	13.5 ± 0.3	26.0 ± 0.5	33.0 ± 0.6	44.2 ± 0.2	21.6 ± 0.2	37.2 ± 0.2
Percent polyenoic	43.4 ± 0.4	43.4 ±0 .3	25.2 ± 0.7	13.0 ± 1.2	36.7 ± 0.3	26.7 ± 0.4
Mean number of double bonds	1.5 ±<0.1	1.3 ±<0.1	1.2 ± 0.1	0.8 ± 0.1	1.4 ±<0.1	1.1 ±<0.1
Mean chain length[c]	18.3 ±<0.1	17.8 ±<0.1	17.8 ±<0.1	17.2 ±<0.1	18.1 ±<0.1	17.6 ±<0.1

[a] Values shown for the experimental diets are after 5 weeks of feeding. Statistical comparison of corresponding lipids of sunflower oil and coconut oil diets revealed statistically significant differences at $p<0.01$ in percent saturated phospholipid and mean chain length of phospholipids categories and at $p<0.001$ in all other cases.

[b] Values shown are mean ± SEM of single determinations on cells from 4-7 individual mice.

[c] Values shown are a weighted mean of number of carbon atoms.

RESULTS

Cell Fatty Acids

Table I shows the fatty acid composition of the phospholipids of the L1210 cells harvested from mice which had been fed the experimental diets for 3, 5, and 7 weeks or commercial mouse chow. There were marked changes in many of the fatty acids, particularly oleic and linoleic acid. The coconut oil diet resulted in an increase in oleate and decrease in linoleate; opposite results were produced by the sunflower oil diet. By contrast, the palmitate and stearate content of the cells changed only slightly. Most of the changes in phospholipid fatty acid composition occurred after the experimental diet was fed for 5 weeks, and little additional change was noted by feeding for an additional 2 week period. The fatty acid composition of the neutral lipids is shown in Table II. The changes brought about by the experimental diets were similar to those found in phospholipids. Since the time of the changes was similar to that of the phospholipids, only the 5 week composition is shown. With each of the diets tested, however, the neutral lipids contained less stearate and arachidonate and more oleate than the cell phospholipids.

The overall changes in the phospholipids

TABLE IV

Content of Major Lipids in L1210 Cells from Mice on Experimental Diets

	Sunflower seed oil diet	Coconut oil diet	Commercial mouse chow
		$\mu g/10^7$ cells[a]	
Phospholipids	62.6 ± 0.9	63.5 ± 0.6	53.7 ± 5.3
Sterols	11.8 ± 0.7	11.6 ± 1.3	12.3 ± 1.7
Acylglycerols	132.2 ± 9.0[b]	95.3 ± 9.7	141.0 ± 27.1
		mole/mole	
Sterol[c]/ phospholipids	0.37 ± 0.02	0.35 ± 0.05	0.44 ± 0.06

[a]Mean ± SEM of single determinations on 5-7 individual mice after 5 weeks of special diets.

[b]Significantly different than the coconut oil diet at $p<0.05$. Otherwise the cells from the sunflower and coconut oil groups did not differ significantly in any of the lipid measurements shown.

[c]Based on molecular weight of cholesterol.

TABLE V

Effect of Diet on Fatty Acid Percentage Composition of Phospholipids and Neutral Lipids in L1210 Ascites Fluid of Mice

	Percentage of fatty acid[a]					
	Sunflower seed oil diet[b]		Coconut oil diet[b]		Commercial mouse chow	
Fatty acid	Phospholipids	Neutral lipids	Phospholipids	Neutral lipids	Phospholipids	Neutral lipids
<14:0	0.6 ± 0.3	0.7 ± 0.3	1.5 ± 0.7	2.9 ± 0.8	0.5 ± 0.2	0.4 ± 0.2
14:0	1.2 ± 0.3	2.0 ± 0.2	1.4 ± 0.2	4.8 ± 0.3	1.0 ± 0.2	1.7 ± 0.4
16:0	23.8 ± 1.4	16.0 ± 0.9	26.0 ± 0.3	14.6 ± 0.7	27.9 ± 1.7	16.9 ± 1.0
16:1	0.4 ± 0.2	4.3 ± 0.3	2.1 ± 0.1	9.1 ± 0.4	0.6 ± 0.4	5.4 ± 0.3
18:0	20.7 ± 1.3	6.5 ± 1.0	15.5 ± 0.2	5.5 ± 0.6	15.2 ± 0.5	5.1 ± 0.9
18:1	11.2 ± 1.5	17.5 ± 1.2	23.9 ± 0.9	22.8 ± 1.7	16.3 ± 0.7	25.8 ± 1.3
18:2	22.5 ± 2.3	34.4 ± 2.3	10.5 ± 0.7	15.1 ± 1.3	19.7 ± 0.6	27.4 ± 2.9
20:1[c]	ND[d]	6.5 ± 1.1	0.3 ±<0.1	6.0 ± 2.6	0.1 ± 0.1	4.5 ± 0.3
20:3	1.0 ± 0.2	0.3 ± 0.1	2.4 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.5 ±<0.1
20:4	8.5 ± 1.0	9.8 ± 1.5	7.5 ± 0.7	14.5 ± 1.3	9.0 ± 0.4	8.8 ± 1.9
22:5	3.5 ± 0.7	0.2 ± 0.1	3.7 ± 0.4	0.6 ±<0.1	1.9 ± 0.3	0.5 ±<0.1
22:6	4.7 ± 0.5	1.2 ± 0.2	2.4 ± 0.4	1.0 ±<0.1	5.4 ± 0.4	2.0 ± 0.4
Others[e]	1.9 ± 0.6	0.6 ± 0.3	2.8 ± 0.5	2.3 ± 0.6	1.3 ± 0.6	1.0 ± 0.3

[a]Values are the percentage composition and are the mean and SEM of single determinations on individual samples from 5-7 mice. The values for ascites plasma of animals on experimental diets are 5 week studies.

[b]The percentage of the following fatty acids in ascites fluid from animals fed the sunflower seed diets were statistically different than fluid from animals fed the coconut oil diet: Phospholipids 16:1, 18:1, 20:1, 20:3 – $p<0.001$; 18:0, 18:2 – $p<0.01$; 22:6 – $p<0.05$. Neutral Lipids 14:0, 16:1, 18:2 – $p<0.001$; <14:0 – $p<0.01$; 18:1, 20:3 – $p<0.05$.

[c]Includes 18:3 and 20:0.

[d]ND = not detected.

[e]Includes small amounts of 20:2, 20:5, 22:0, and unidentified fatty acids.

can best be appreciated from Table III. Although the percentage of total saturated or unsaturated fatty acids did not differ greatly among the cells grown on the three diets, there was a marked shift in the composition of the unsaturated fatty acids. Tumor cells from mice fed the unsaturated sunflower oil diet for 5 weeks contained 18% more polyenoic, 38% less monoenoic fatty acids, and a larger mean number of double bonds per fatty acid than when the animals were fed commercial chow. In contrast, the cells from animals fed the coconut oil diet which is composed of fatty acids of greater saturation had 31% less polyenoic fatty acids and a smaller mean number of double bonds compared to animals fed the commercial chow. There was a slightly higher percentage of saturated fatty acids contained in the phospholipids of the tumor cells from animals fed the sunflower oil diet. This seemingly paradoxical change has been noted in dietary studies with mouse depot fat, rat liver mitochondria, and Ehrlich ascites tumor cell plasma membranes (6,14,15). It has been suggested that this increase in saturated fatty acids may be a compensation for the increase in polyenoic fatty

acids, thereby maintaining membrane fluidity (6). In this regard, there is evidence that the microsomal desaturation of fatty acids may be a regulated process (16,17). Holloway and Holloway have shown that mouse liver microsomes from animals on an unsaturated fat-rich diet contained more stearate and less stearyl coenzyme A desaturase activity than animals on a fat-free diet (18). It seems likely that under certain conditions, the polyunsaturated fatty acids may inhibit the desaturation of the major saturated fatty acids. Unlike the phospholipids, however, the proportion of saturated fatty acids of the neutral lipids was lower in cells from mice on the sunflower oil diet.

Cell Lipid Content

The content of sterols, phospholipids, and acylglycerols of the L1210 cells are shown in Table IV. There was no major difference in sterols and phospholipids between cells from animals fed the sunflower and coconut oil diets. Likewise, the sterol to phospholipid ratio was similar. The cell content of acylglycerols was lower in the cells from animals fed the coconut oil diet as compared to cells from animals fed the sunflower oil diet ($p<0.05$).

Ascites Fluid Fatty Acids

The fatty acid composition of the phospholipids and neutral lipids of the ascites fluid in which the L1210 cells grow in the mouse peritoneal cavity is shown in Table V. These values are for fluid obtained after 5 weeks of feeding. The differences in fatty acid composition produced by the diets are similar to those observed for the cell lipids. Compared to the L1210 cell, the ascites fluid neutral lipids contained a higher percentage of polyenoic fatty acids, especially linoleate, and a greater mean number of double bonds regardless of the diet. In additional studies, we observed that the fatty acid composition of the ascites fluid at 3 and 7 weeks was also similar to that of the L1210 cells at these times. This suggests that the dietary changes in the fatty acids of the cells and ascites fluid are interrelated processes.

DISCUSSION

Considerable changes in the fatty acid composition of the L1210 murine leukemia cells occurred when the mice were fed diets that contained different fats. Changes were observed in both the phospholipids and neutral lipids. The changes in neutral lipids might be anticipated since most of this fraction is composed of triglycerides, a storage form of lipid. It may make little difference to the cell integrity and function as to exactly what type of fatty acid is stored in triglyceride droplets. In contrast, the appreciable changes in cell phospholipids are more likely to have important functional implications. Most cell phospholipids are contained in membranes. A greater degree of unsaturation of the fatty acyl groups of the phospholipids, such as occurred in the cells obtained from the mice fed sunflower oil, should lead to increased membrane fluidity and greater permeability (19). This could be compensated for by changes in either the sterol/phospholipid ratio or the fatty acid chain length. Our data indicate that the sterol/phospholipid ratio was not modified appreciably by either of the test diets. The mean fatty acid chain length was statistically greater in the cells on the sunflower diet but the numerical differences appear small and their biologic effect is questionable. Taken together, these observations suggest that the diet-induced changes in fatty acid composition of the L1210 cell phospholipids probably lead to alteration in the membrane fluidity of the cells.

Diet-induced changes have been produced in other tumors. Wood studied the effect of a fat-free diet on the lipid composition of 7288 CTC rat hepatoma (3). He found changes in the fatty acid composition of the major phospholipids and showed that these changes occurred in the fatty acids of the 2-position of the phospholipids, not in fatty acids of the 1-position. Liepkalns and Spector studied the fatty acyl composition of the cellular lipids of Ehrlich ascites tumor cells carried by mice which were fed diets rich in either polyunsaturated or saturated fats (2). Large alterations were produced in the fatty acyl composition of the phospholipids. These modifications of cell phospholipids were similar to those of the L1210 cells.

It is known that the fatty acid composition of human platelets and erythrocytes can be altered by changing the saturation of the diet (20,21). The equilibrium between dietary and erythrocyte fatty acid composition occurred after 4-6 weeks, and it was suggested that the exchange occurred through a precursor pool of fatty acids in plasma or elsewhere in extracellular spaces (20). The fatty acid composition of the plasma lipoproteins of humans can be altered by changing the saturation of the dietary fat intake (22,23). Therefore, it is likely that the plasma lipoprotein lipids represent this intermediary pool. In our study, the lipid esters of the ascites fluid underwent an alteration of fatty acid composition that was similar to the change in the L1210 cell itself. It seems likely that there is an equilibrium established between the lipids of the leukemia cell and those of its suspending medium such that modifications of

their fatty acid composition occur together. One can speculate that an alteration of a precursor pool of body fat and of plasma lipoproteins occurs prior to the modification of ascites fluid and tumor cell.

The tumors in which lipid modifications have been studied previously have importance as examples of neoplastic processes. However, observations with these tumors may have limited implication for human neoplasia. On the other hand, the L1210 murine leukemia is one of the major testing models used by the National Cancer Institute for initial screening of antineoplastic drugs (4). Most of the clinically useful antineoplastic drugs have activity against the L1210 leukemia and conversely, many agents with little clinical utility are inactive against the murine leukemia (4). Therefore, it would be worthwhile to explore the possible thereapeutic implications of fatty acid modifications using this experimental tumor system. This system is a logical one in which to test the effect of fatty acid modification on sensitivity to cytotoxic drugs, since drug responsiveness in the L1210 leukemia is often predictive of drug responsiveness of human leukemia.

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Antimicrobial Lipids: Natural and Synthetic Fatty Acids and Monoglycerides

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ABSTRACT

Over 40 natural or synthetic lipophilic compounds were screened for antimicrobial activity. Gram (+) bacteria and yeasts but not Gram (-) bacteria were affected by these agents. Epimino and selena fatty acids are more active than their corresponding straight chain unsubstituted fatty acids. The position of selenium influenced the antimicrobial activity of the fatty acid. The presence and position of a double or triple bond, usually an important factor in long chain fatty acids ($>C_{14}$) had little or no effect in C_{11} fatty acids. Optimum antimicrobial activity was found for fatty acids and their corresponding monoglycerides when the chain length was C_{12}. The dilaurin derivative was not active.

INTRODUCTION

Previously, we have reported on the structure-function (antibacterial activity) relationship for fatty acids (1,2) and polyol esters (3). Much of the early literature on this subject can be found in reports by Bayliss (4), Kodicek (5), and Nieman (6). These studies showed that the optimum activity for even carbon numbered aliphatic fatty acids was found in C_{12} saturated fatty acids and C_{18} mono- and diunsaturated fatty acids. While esterification of these fatty acids with monohydric alcohols leads to inactive species, esterification to polyhydric compounds gave derivatives which were more rather than less active (3).

This paper describes the antimicrobial activity of other common and uncommon fatty acids and affords a closer definition of structure-function relationships for monoglycerides. Compounds used in this report were selected during the course of investigating relatively nontoxic antimicrobial agents for possible use in food, cosmetics, and pharmaceutical industries.

MATERIALS AND METHODS

Sources of Lipophilic Agents

The C_{11} unsaturated series of acids were prepared by methods previously reported (7). Eight brassylic acid derivatives were sent for testing by Shu-Pei Chang, USDA, Northern Regional Research Laboratory (8), A. Fredga, Uppsala, kindly provided the selena acids. T.A. Foglia, USDA, Eastern Regional Research Center, furnished some epimino derivatives (internal aziridines) (9). The fatty acids and monoglycerides used in this study were synthesized and chromatographically purified by one of the authors (M.S.F. Lie Ken Jie).

Standard suspensions of the lipophilic materials were prepared (10-12) by first dissolving the weighed compounds in 1-2 ml of 95% alcohol or methanol. Trypticase Soy Broth (TSB) was added to the dissolved or suspended compound and diluted to appropriate concentrations.

The specifics of the procedure and the microorganisms used in this study are those previously reported (1-3). The organisms have been continuously maintained and checked in our laboratory. Exceptions are listed in Table V. These organisms were used in confirmatory experiments conducted by W.A. Zygmunt (Mead Johnson Research Center, Evansville, IN).

The inoculum (0.05 ml of an 18-24 hr TSB culture containing ca. 10^8-10^9 organisms per ml) was aseptically delivered to all assay tubes. All cultures were incubated at 36 C with the exception of the dermatophytes, for which a 28 C temperature was employed. Inoculated broth without compound served as a positive control. An uninoculated set of dilutions with the test compounds served as a negative control.

The minimal inhibitory concentration (MIC) of each compound was determined for each test organism. The MIC is defined as the lowest concentration of compound at which no macroscopic evidence of growth was observed after 18 hr of incubation (7 days with the dermatophytes).

In those cases in which the test compound itself caused appreciable turbidity so that the MIC could not accurately be determined, a sample (0.1 ml) of the mixed broth in question was inoculated on a Trypticase soy agar plate containing 5% defibrinated sheep blood, incubated at 36 C and examined after 24 hr for

TABLE I

MIC[a] (μg/ml) of Epimino Derivatives

Derivative	Organisms						
	Gram (+)				Yeasts		
	Sf	Sp	Sa	Csp	Na	Ca	Sc
cis-9, 10-epimino octa decan-1-ol R_1-$(CH_2)_7CH_2OH$[c]	NI[b]	NI	100	100	100	NI	100
cis-9, 10-epimino octa decanoate-K R_1-$(CH_2)_7C^{C}_{OK}$	NI	NI	1,000	1,000	1,000	1,000	2,000
cis-9, 10-epimino octadecane R_1-$(CH_2)_7CH_3$	NI	1,000	100	100	1,000	NI	NI
trans-9, 10-epimino octadecane	NI	1,000	1,000	1,000	1,000	NI	1,000
Stearic acid-$C_{18:0}$	NI	NI	NI	NI	NI	NI	NI
Oleic acid-$C_{18:1}$	500	NI	NI	NI	NI	NI	NI

[a]Abbreviations: MIC = minimal inhibitory concentration; Ec = *E. coli;* Pa = *P. aeruginosa;* Sf = *S. faecalis;* Sp = *S. pyogenes;* Sa = *S. aureus;* Csp = *Corynebacterium* Sp; Na = *N. asteroides;* Ca = *C. albicans;* Sc = *S. cerevisiae.*

[b]Ni = MIC >1,000 μg/ml (*E. coli* and *P. aeruginosa* [gram (-)]) were not affected at 1,000 μg/ml.

[c]$R_1 = CH_3(CH_2)_7-\overset{H}{\overset{\diagup N \diagdown}{C}}-\overset{H}{C}-$

TABLE II

Selena Substituted Fatty Acids MICs[a] μg/ml

Organism	Fatty acid[b]					
	$C_{14}Se^{10}$	$C_{15}Se^{4}$	$C_{15}Se^{6}$	$C_{17}Se^{5}$	$C_{17}Se^{6}$	Palmitic[c] acid
Streptococcus faecalis (Group D)	1,000	NI[d]	1,000	NI	1,000	1,000
Streptococcus pyogenes	100	100	10	NI	1,000	1,000
Staphylococcus aureus	1,000	NI	100	NI	NI	NI
Corynebacterium sp.	100	1,000	10	NI	1,000	1,000
Norcardia asteroides	100	1,000	10	1,000	100	NI
Candida albicans	100	NI	NI	NI	NI	NI
Saccharomyces cerevisiae	100	NI	1,000	NI	NI	NI

[a]MIC = minimal inhibitory concentration.

[b]*E. coli* and *P. aeruginosa* [Gram] were not affected at 1,000 μg/ml.

[c]Stearic acid (C_{18}) was inactive at 1,000 μg/ml.

[d]NI = MIC >1,000 μg/ml.

the bactericidal end point. There was usually only a one or two tube dilution difference between biocidal and biostatic concentrations.

RESULTS

Dicarboxylic Acid Polyesters

Brassylic acid (C_{13}) and other straight chain dicarboxylic acids were esterified via polyesterification and tested for antimicrobial activity. Neither brassylic acid or its corresponding oligomeric diesters previously reported (8) were active at levels of 1,000 μg/ml against any of the test organisms.

Epimino Derivatives

A small series of epimino derivatives (R_1 $\overset{H}{\overset{\diagup N \diagdown}{C}}-\overset{H}{C}-R_2$), internal aziridines, representing a hydrocarbon, alcohol, and acid derivative, were provided for testing (9). In addition, the biological effect of a *cis* and *trans* isomer was compared. Results provided in Table I indicate that the order of increasing activity was hydrocarbon <acid<alcohol. The *trans* hydrocarbon was less active than the *cis* isomer. Stearic and oleic acids are not active under these condi-

tions. These data are provided for comparative purposes only.

Selena Substituted Fatty Acids

A number of fatty acids containing selenium in place of a CH_2 group were screened. Despite the few compounds tested, data in Table II allow a few generalizations. The shorter selena fatty acid (C_{15}) (10-selena-pentadecanoic acid, $C_{14}Se^{10}$) is more active than the C_{16} or C_{18} unsubstituted fatty acids; addition of selenium to the chain increases the antimicrobial activity of the fatty acid; selenium is more effective when positioned furthest from the carboxyl group of the fatty acid (e.g., $C_{15}Se^{6}$ was more active than $C_{15}Se^{4}$; $C_{17}Se^{5}$ was less active than $C_{17}Se^{6}$).

The unsubstituted saturated fatty acids (C_{16}, C_{18}) were less active than the selena substituted fatty acids.

Unsaturated C_{11} Fatty Acids

These highly purified liquid fatty acids were available only in small quantities; and, therefore, MIC values are based on volume rather than weight. Position of ethylenic unsaturation makes very little difference to C_{11} antibacterial activity. The acetylenic derivatives showed greater differences in respect to position of unsaturation (Table III). Both the $C_{11}\Delta^{6a}$ and $C_{11}\Delta^{10a}$ were more active (MIC = 250 μg/ml) than the other acetylenic derivatives isomers (MIC >500 μg/ml).

It should be noted that the saturated C_{11} acid is more active than the unsaturated derivatives and has an MIC value of 125 μg/ml against *Streptococcus pyogenes.*

Fatty Acid vs. Monoglyceride

In order to define more closely the effect of esterification on fatty acid antimicrobial activity, C_{11}, C_{12}, and C_{13} fatty acids and their corresponding monoglycerides were examined. Table IV contains results of such a screen. Of the saturated fatty acids, the C_{12} acid was more active than the C_{11} compound which was more active than the C_{13} acid; but the $C_{13}\Delta^{13e}$ unsaturated fatty acid was more active than the C_{13} saturated fatty acid. In the lower series, $C_{11}\Delta^{10e}$ and $C_{12}\Delta^{11e}$ were less active than their corresponding saturated acids.

Esterification of a fatty acid to glycerol to form a monoacyl derivative generally results in more active derivatives. This effect was most pronounced in the C_{12} series, where the most active ester was monolaurin.

The undecynoyl ($C_{11}\Delta^{10a}$) monoglyceride was slightly more active than the undecenoyl ($C_{11}\Delta^{10e}$) monoglyceride.

TABLE III

MIC[a] Values (μg/ml) for Unsaturated C_{11} Fatty Acids Against *Streptococcus pyogenes*

Position of unsaturation	Undecenoate[b] values	Undecynoate values
Δ^2	NI	1,000
Δ^3	500	500
Δ^4	500	500
Δ^5	500	500
Δ^6	500	250
Δ^7	500	1,000
Δ^8	500	500
Δ^9	500	500
Δ^{10}	1,000	250

[a]MIC = minimal inhibitory concentration.

[b]Undecanoic acid = 125 μg/ml.

The principle of maximizing the activity of an aliphatic fatty acid by esterification to a polyol was repeated in Table V. Polyols other than glycerol are generally not as active.

Activity of the monolaurin can best be seen by its effect on the growth curve of *S. pyogenes* (Fig. 1). While 5.0 μg/ml has a slight bacteriostatic effect, 10.0 μg/ml almost completely prevents growth for 24 hr. It should be noted that at the same level the diglyceride (dilaurin) has no antibacterial effect.

In reference to pH, the monolaurin is most active at high (8.0) and low (5.0) pH values (Table VI). Activity seen at low pH, however, may be due to poor growth conditions as well as compound activity. This is true more for bacteria than for yeast.

DISCUSSION

The present study confirms the well-known antimicrobial effect of lipids and expands our previous findings on the subject (1-3). Such screening experiments provided us with greater insights to structure-function relationships and confirm the usefulness of these compounds as nontoxic preservatives (2,10).

Brassylic acid and its corresponding diesters were all inactive at concentrations of 1,000 μg/ml. This confirmed previous findings that long chain dicarboxylic acids are inactive as antimicrobial agents (1). Diesters are also known to be less active than monoesters (3).

In internal aziridine (epimino) fatty acids, the addition of a nitrogen across the double bond forms a derivative more active than either the corresponding saturated or unsaturated aliphatic fatty acid. The alcohol derivative in the aziridine series being more active than the fatty acid and the *cis* isomer being more active than the *trans* confirms our previous report for

TABLE IV

MIC[a] Values (μg/ml) for Fatty Acids and Their Corresponding Monoglycerides

Organism[b]	Compound				
	Undecanoic acid	10-Undecenoic acid	10-Undecenoyl monoglyceride	10-Undecynoyl monoglyceride	11-Dodecenoic acid
Streptococcus faecalis (Group D)	NI[a]	NI	500	500	NI
Streptococcus pyogenes	125	1,000	125	125	250
Staphylococcus aureus	1,000	1,000	500	500	NI
Corynebacterium sp.	31	31	62	62	125
Norcardia asteroides	62	125	125	62	62
Candida albicans	1,000	1,000	250	100	1,000
Saccharomyces cerevisiae	500	500	250	100	500
	Dodecanoic acid	Dodecanoyl monoglyceride	12-Tridecenoic acid	Tridecanoic acid	Tridecanoyl monoglyceride
Streptococcus faecalis (Group D)	500	NI	1,000	NI	NI
Streptococcus pyogenes	62	8	125	1,000	62
Staphylococcus aureus	500	250	1,000	NI	NI
Corynebacterium sp.	31	16	31	NI	NI
Nocardia asteroides	62	16	125	1,000	125
Candica albicans	1,000	500	1,000	NI	NI
Saccharomyces cerevisiae	1,000	250	500	1,000	NI

[a]MIC = minimal inhibitory concentration.

[b]*E. coli* and *P. aeruginosa* were not affected at 1,000 μg/ml.

[c]NI = MIC >1,000 μg/ml.

TABLE V

Antimicrobial Activity of Fatty Acids and Monoglycerides[a]

	Capric acid	1-Monocaprin	Sucrose caprate	Lauric acid	1-Monolaurin	(Tri)glycerol monolaurate
M. audouini 9079	12.5	50	200	100	12.5	100
M. canis 10241	12.5	50	400	100	6.25	100
T. mentagr. (gypsium) 9129	25	25	400	100	12.5	100
T. mentagr. (interdigitale) 9972	12.5	25	400	100	6.25	100
T. mentagr. (asteroides) 8757	25	25	400	100	12.5	50
C. albicans 10231	100	200	>400	>400	>400	>400

[a]Minimal inhibitory concentration = μg/ml. Data provided by W.A. Zygmunt.

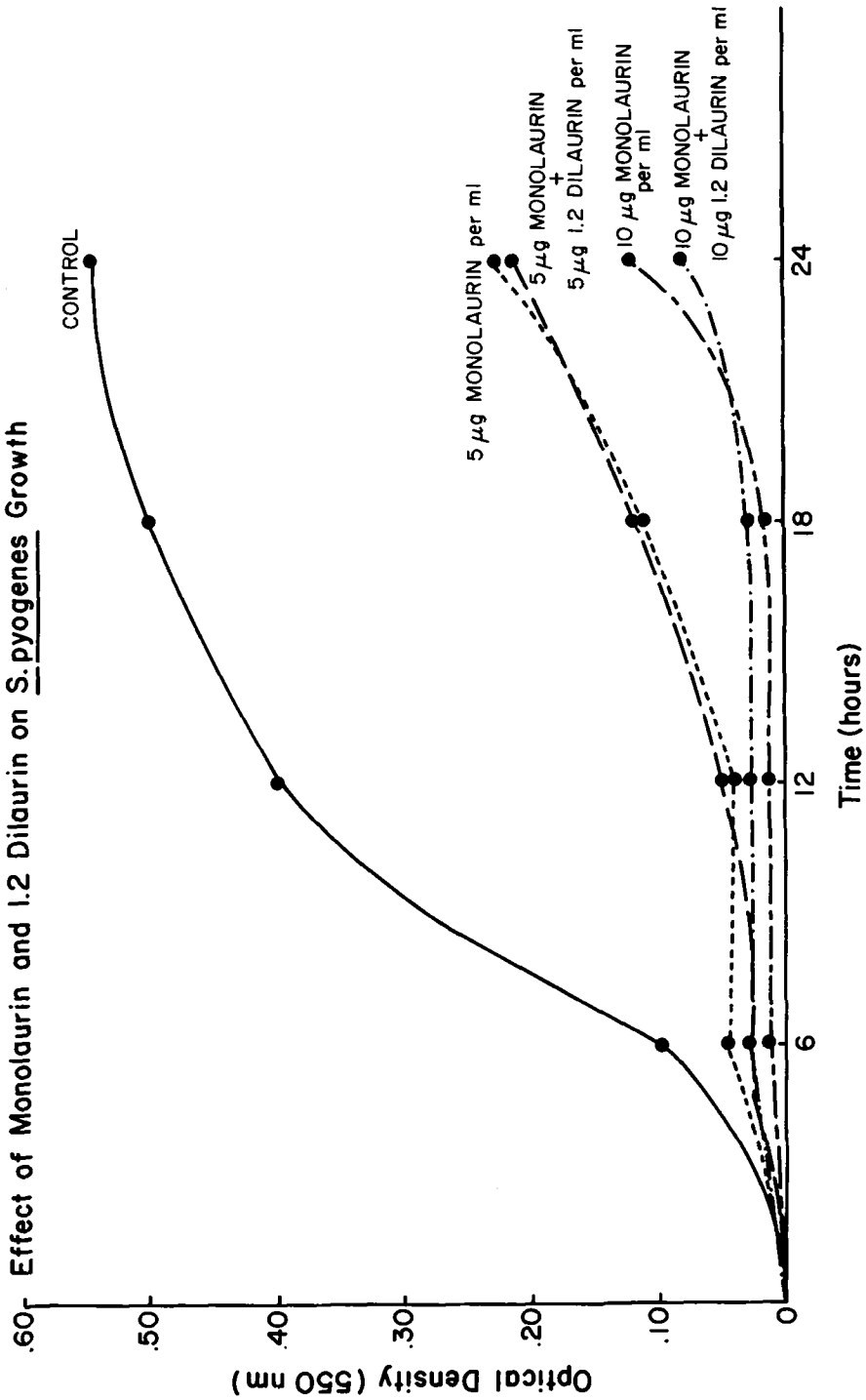

FIG. 1. Growth of *S. pyogenes* in TSB broth was followed by change in optical density at 550 nm with and without antimicrobial lipid, monolaurin.

TABLE VI

Effect on pH on MICs[a] (μg/ml) of Monolaurin

Organism	pH 5.0	pH 6.0	pH 7.0	pH 8.0
Escherichia coli	>1,000	>1,000	>1,000	>1,000
Pseudomonas aeruginosa	500	>1,000	>1,000	>1,000
Streptococcus mutans	15.6	31.2	31.2	31.2
Streptococcus agalactiae	15.6	15.6	15.6	15.6
Staphylococcus aureus	15.6	62.5	250	62.5
Corynebacterium sp.	(-)[b]	(-)[b]	15.6	15.6
Candida albicans	>1,000	>1,000	>1,000	>1,000
Saccharomyces cerevisiae	15.6	250	500	125

[a]MIC = minimal inhibitory concentration.

[b]No growth in control TSB blanks at the indicated pH.

such generalizations (1). The antimicrobial activity of the epimino hydrocarbon compared favorably with that exhibited by the acid and suggests that other hydrocarbons be investigated further as potential inhibitors. Squalene, however, was found not to be active in our preliminary screening tests (unpublished results).

Interest in the effects of unsaturation on biological activity led us to study selenium substituted fatty acids. Selenium forms bonds similar to carbon except the bond angle between adjacent carbons is smaller. This in effect put a kink in the aliphatic chain. Similar bending in *cis* unsaturated isomers appears to increase the antimicrobial effect of a normally straight chain compound (2). Also, the bend in the chain causes the fatty acid to be more active if the Se is located in the Se^6 position as compared to the 4 or 5 position. Whether this represents a specific position in the chain for maximum biological activity cannot be deduced because of the limited number of acids tested.

Again, the present paper reaffirms our earlier conclusion on the importance of unsaturation to fatty acid antimicrobial activity, i.e., unsaturation contributes little biological activity to short chain fatty acids ($<C_{12}$). While the position of unsaturation was shown to be important for $C_{12:1}$ and $C_{18:1}$ fatty acids (11), our present data for $C_{11:1}$ gave no such relationships. However, as found previously, acetylenic compounds were slightly more active than ethylenic isomers. As in the case of $C_{12:1}$ fatty acids, both isomers of the $C_{11:1}$ were less active than their saturated counterparts.

The antimicrobial activity of C_{11} and C_{13} fatty acids as monoglycerides showed both to be less active than monolaurin. In general, lower chain fatty acids ($<_{16}$) are biologically more active when mono esterified to glycerol. This principle is best exemplified with glycerol although lauric esters of other polyhydric alcohols are also active (3).

Monolaurin remains the most active simple lipid screened to date. The presence of dilaurin (diester) has no effect on the antimicrobial activity of monolaurin (Fig. 1). The addition of a closely related monoglyceride like monomyristin or monopalmitin, however, lowers activity of the monolaurin derivatives. This is in contrast to other antimicrobial compounds of mixed chain length which are more active than compounds with a single chain length (11,12).

Since compounds like monolaurin may have utility mainly as surface active agents, it is fortunate that the antimicrobial action is strongest in acidic environments. Skin (pH 5.5) and saliva (pH 6.0-6.5) are environments where optimal antimicrobial action of monolaurin can be achieved.

It should be emphasized that the majority of fatty acids and derivatives are most effective against Gram (+) bacteria and yeasts. Except for certain short chain fatty acids, Gram (-) organisms are generally not affected (13). These facts suggest that the mechanism of bactericidal action of long chain fatty acids and derivatives is due to a balance between hydrophilic and hydrophobic parts of the molecule. The hydrophobic portion of the molecule is the more important constituent since dodecanoic derivatives either as alcohols, aldehydes, carboxylic acids, amides, amines, or aminimides are all active (1-3,10,12,14). Regardless of the polarity of the hydrophilic portion, optimum chain length is between C_{12} and C_{16}. The shorter chain is more important for neutral and acidic hydrophilic groups while cationic and dipolar compounds have optimum activities at the longer chain lengths (C_{14}, C_{16}).

The answer to why Gram (-) bacteria are less sensitive to lipophilic agents can be found in the complexity of their cell walls. Galbraith and Miller (15), Sheu and Freese (13), and more

recently, Kondo and Kanai (16) have discussed similar mechanisms of action. All these findings suggest that the fluidity of the cell membrane can be disturbed maximally by lipophilic compounds of particular chain lengths, unsaturation (bending) or with particular hydrophilic functional groups. The functional group playing an important but as yet undefined role in terms of surface chemistry. Contrary to finds with long chain fatty acids or amides, long chain compounds with a basic group (NH_2) are active against Gram (-) bacteria (14,17).

If one considers the addition of these simple lipid derivatives as messages to which the organism can or cannot respond to in varying degrees, it may be possible to learn more about the surface chemistry of cells by the use of such agents. Pheromones, simple lipids, are prime examples by which such communication is possible (18). It is not beyond imagaination for us to communicate to a whole host of cell types by such agents in order to control their function. Understanding and control of the "braille of cellular surfaces" becomes biology's biggest challenge.

ACKNOWLEDGMENTS

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[Received March 14, 1977]

SHORT COMMUNICATIONS

Stereospecific Synthesis of *cis* and *trans* Fatty Esters

ABSTRACT

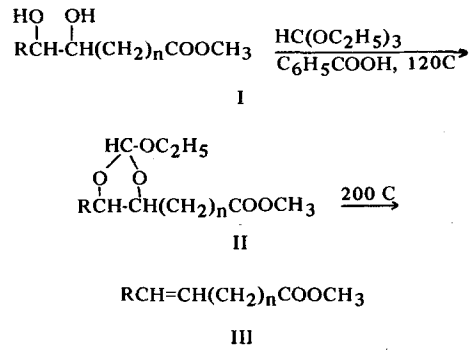
The *erythro* and *threo* isomers of methyl 9,10-dihydroxyoctadecanoate and the *threo* isomer of methyl 12,13-dihydroxy-*cis*-9-octadecenoate were converted into methyl *cis*- and *trans*-9-octadecenoate and methyl *cis*-9,*trans*-12-octadecadienoate, respectively, by reaction of the dihydroxy ester with triethyl orthoformate to give the 2-ethoxy-1,3-dioxolane which was thermally decomposed to the unsaturated ester.

INTRODUCTION

1,2-Diols have been converted stereospecifically into alkenes via dibromides (1), benzylidene acetals (2), cyclic thiocarbonates (3-7), dimesylates (8), and by direct reaction with phosphonium iodide in acetic acid (9). Gunstone and Jacobsberg (10) have successfully applied two of these reactions to the fatty acid field. They were able to convert methyl *threo*-12,13-dimesyloxyoleate to methyl *cis*-9,*trans*-12-octadecadienoate in 58% conversion (80% yield after allowing for unreacted dimesylate). Yield is not given, however, on the preparation of the dimesylate. The 9*c*,12*t*-18:2 ester was also obtained from methyl *threo*-12,13-dihydroxyoleate through the cyclic thiocarbonate but no yields are given for this route.

RESULTS AND DISCUSSION

We have successfully applied a reaction first described by Crank and Eastwood (11) and Josan and Eastwood (12) which is convenient and simple to carry out, uses readily available reagents, and gives better than 80% conversion of the diol to the desired alkene. In this reaction, the dihydroxy ester, I, is heated slowly with triethyl orthoformate and an acid catalyst to form ethanol and the 2-ethoxy-1,3-dioxolane, II. This product may be isolated, if desired, by distillation from a basic solution. However, for preparing the alkene, it is only necessary to heat the unisolated intermediate slowly to about 200 C, at which point it decomposes to ethanol, CO_2, and the unsaturated ester, III.



We have applied this reaction to the stereospecific conversion of methyl *erythro*- and *threo*-9,10-dihydroxystearates into methyl oleate and elaidate, respectively, and methyl *threo*-12,13-dihydroxy-*cis*-9-octadecenoate into methyl *cis*-9,*trans*-12-octadecadienoate.

Silver ion thin layer chromatography (Ag-TLC) and gas liquid chromatography (GLC) on OV275 were used for *cis-trans* separation and identification. Retention times for the several isomers were determined with authentic samples and compared well with the results obtained.

^{13}C nuclear magnetic resonance (NMR) spectroscopy also served to demonstrate the identity and purity of the products. The chemical shift is about δ27.3 for carbon atoms alpha to a *cis* double bond and about δ32.7 for carbon atoms alpha to a *trans* double bond. The oleate prepared here showed a peak at δ27.23 and none at δ32.7, while the elaidate showed a peak at δ32.69 and none at δ27.23. Analyses of weighed mixtures indicated that less than 4% of one isomer could be detected in a mixture with the other isomer.

In the 9,12-diene, the pertinent chemical shifts were C8 δ27.25; C11, δ30.55; C14, δ32.70. The chemical shift for a methylene group between two double bonds is δ25.75 if both are *cis*, δ35.70 if both are *trans*, and δ30.55 if one double bond is *cis* and the other is *trans* (13). Thus, this diene is 9-*cis*,12-*trans*.

EXPERIMENTAL PROCEDURE

Gas liquid chromatography was conducted on a Packard 7400 series gas chromatograph with a flame ionization detector. The glass

column (4 mm x 20 ft) was packed with 15% OV275 on 100/120 mesh Gas Chrom P-AW-DMCS and operated at 200 with a flow of 35 ml/min of He at a pressure of 37 psi.

Thin layer chromatography was carried out on Precoated TLC Silica Gel 60 F254 plates (E. Merck) impregnated with silver nitrate, and developed in 100% benzene.

^{13}C NMR spectroscopy was conducted on a Bruker WH 90 Fourier Transform NMR spectrometer operating at 22.63 MHz.

Methyl *trans*-9-Octadecenoate

threo-9,10-Dihydroxystearic acid (mp 94 C) was converted with methanol and sulfuric acid to the methyl ester in 95% yield. In a small distillation apparatus were placed methyl *threo*-9,10-dihydroxystearate (0.8 g, 0.0024 mole), triethyl orthoformate (0.8 g), and benzoic acid (0.03 g). The pot was heated slowly in an oil bath to 120 C and the theoretical amount of ethanol was collected. The pot was then heated slowly to 210 C and all volatile material was collected. After cooling to room temperature, the pot contents were passed through a column (15 mm ID) packed with silica gel (5 g). The first 50 ml of petroleum ether eluate contained 0.68 g (0.0023 mole, 95% yield) of methyl elaidate. This material gave one spot on Ag-TLC and showed about 4% of ethyl benzoate and no methyl oleate on GLC.

Methyl *cis*-9,*trans*-12-Octadecadienoate

Vernonia anthelmintica seed oil was acetolyzed, saponified, and esterified as described by Gunstone (14). The mixture of *Vernonia* methyl esters was separated on a silica gel chromatographic column into a nonhydroxy fatty ester fraction (21%) (PE:EE-90:10) and methyl *threo*-12,13-dihydroxy-*cis*-9-octadecenoate (79%) (PE:EE-50:50). This dihydroxy ester (3.8 g, 0.0115 mole) was placed in the pot of a small distillation apparatus together with triethyl orthoformate (1.8 g, 0.0122 mole) and benzoic acid (0.05 g). The pot was heated slowly in an oil bath to 120 C and kept at this temperature for 0.5 hr while distillate was collected. The temperature was then raised slowly to 210 C and all volatile material was collected. The pot contents were passed through a column (15 mm ID) containing silica gel (8 g). The first 100 ml of petroleum ether eluate contained 2.77 g (82%) yield) of methyl *cis*-9,*trans*-12-octadecadienoate. GLC showed the presence of about 1.5% ethyl benzoate and no methyl linoleate. Ag-TLC showed one spot which ran a little ahead of methyl linoleate and a little behind methyl oleate.

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Lipogenesis in the Developing Brain from Intracranially Administered [1-^{14}C] Acetate and [U-^{14}C] Glucose

ABSTRACT

Fifteen-day-old rats divided into two groups were given [1-^{14}C]acetate or [U-^{14}C]glucose by intracranial injection and were sacrificed after 1 hr. Analysis of lipids from the two groups showed differences in the incorporation of radioactivity in the polar lipids and cholesterol. Analysis of brain fatty acid showed that whereas radioactivity from acetate was incorporated into saturated, mono- and polyunsaturated fatty acids, the radioactivity from [U-^{14}C]glucose was found only in 16:0, 18:0, and 18:1. No radioactivity was found in polyunsaturated fatty acids even after concentration of this fraction by $AgNO_3$:SiO_2 thin layer chromatographic method. This difference is discussed in hypothetical terms of nonhomogeneous acetyl CoA pool, formation of acetyl CoA from glucose exclusively inside the mitochondria, and activation of injected acetate to acetyl CoA.

INTRODUCTION

Previous work from this laboratory (1) indicated that acetyl CoA produced by β-oxidation of palmitate was mostly used for synthesis of cholesterol and chain elongation of endogenous fatty acids rather than de novo synthesis of palmitate itself. Externally administered acetate, however, was rapidly used not only for de novo synthesis of palmitate but for all other reactions of lipogenesis (2). Acetyl CoA, which is a pivotal precursor for fatty acid biosynthesis, can be generated by glycolysis and oxidation of glucose, β-oxidation of fatty acids or oxidative reactions of certain amino acids (3,4). Recently, utilization of ketone bodies for fatty acid and sterol synthesis has been reported (5,6). This work was undertaken to study the utilization of acetyl CoA produced from radioactive glucose and to compare it with exogenously administered [1-^{14}C]acetate for lipogenesis in the developing brain.

MATERIALS AND METHODS

Sixteen 15-day-old Wistar, male and female, rats weighing 26 ± 2 g were divided into group A (10 rats) and group B (6 rats). [1-^{14}C]-sodium acetate (45-60 mCi/mM) and [U-^{14}C]-glucose (150-250 mCi/mM) (New England Nuclear, Boston, MA.) were used. Group A received 50 μCi of [1-^{14}C]sodium acetate per rat, and group B received 33.3 μCi of [U-^{14}C]-glucose per rat by an intracranial injection of an aqueous solution (7). All animals were sacrificed 1 hr after tracer injection.

The brain was excised following decapitation, and lipids were extracted from the pooled tissue by the Folch et al. method (8). Fractionation was achieved by combination of SiO_2 column and TLC, and radioactivity determined as described previously (9). Fatty acid methyl esters were obtained by methanolysis using 1% H_2SO_4 in methanol. Total fatty acids were separated into classes according to the degree of

TABLE I

Percent of Radioactivity in Various Lipid Components Folloiwng Intracranial Injection of [1-^{14}C]Acetate and [U-^{14}C]Glucose

	1 hr after [1-^{14}C]acetate	1 hr after [U-^{14}C]glucose
(% TL Radioactivity)		
Total polar lipids[a]	63.9	74.2
Free cholesterol	23.0	14.7
Total acyl glycerides	5.2	5.6
Free fatty acids	3.1	4.4
(% Total polar lipids)		
Phosphatidyl choline (PC)	58.3	55.0
Phosphatidyl ethanolamine (PE)	20.4	27.5
Phosphatidyl serine (PS)	10.4	2.2
Cerebroside + sphingomyelin	10.0	12.5

[a]Polar lipids = total phospho- and spingolipids; TL = total lipids.

TABLE II

Percent Disgribution of Radioactivity in Major Brain Fatty Acids Following [1-^{14}C] Acetate and [U-^{14}C] Glucose Injection

Fatty acid	1 hr after [1-^{14}C] acetate	1 hr after [U-^{14}C] glucose
14:0	2.7	–
16:0	56.1	65.3
18:0	19.5	21.3
18:1	12.2	13.4
20:4	9.5	–
22:6	1.8	–

[a]The specific activity of fatty acid methyl esters after acetate injection was 68,303 cpm/mg and after glucose injection was 6,128 cpm/mg; 0.3 mg was injected in the former and 3.0 mg was injected in the latter gas liquid chromatographic (GLC) run. Attenuation for radioactivity was increased to give full scale deflection for 650 cpm.

unsaturation by using $AgNO_3$:SiO_2 TLC as described before (7). Decarboxylation of pure fatty acids by the Schmidt method was carried out as described by Brady et al. (10).

Radio gas liquid chromatography (GLC) was performed using a Packard GLC Model 824 in combination with a proportional counter Packard Model 894 under conditions analogous to those described earlier (7). This instrument gives full scale deflection for radioactivity of 1,300 cpm at a setting of 1 K, but the instrument can be used at double this sensitivity. When the retention time of any particular peak is long, this fraction is hydrogenated. The corresponding saturated fatty acid peak gives a sharp distinct peak.

RESULTS

One hr after injection of [1-^{14}C] acetate, 2.75% of the injected radioactivity (2.57×10^6 cpm) was retained in the brain, whereas a smaller amount, 0.75% (468,343 cpm), was retained in the brain following [U-^{14}C] glucose administration. The percent distribution of radioactivity in various neutral and polar components separated by TLC in the brain following injection of the two tracers is shown in Table I. The polar lipids contained a higher portion of the total radioactivity after glucose injection than after acetate injection. This could be due to the fact that some of the radioactivity from radioactive glucose could be incorporated into the glycerol backbone of the phosphlipids. Cholesterol, on the other hand, contained less radioactivity after glucose injection than after acetate injection. Phosphatidyl choline (PC) had the highest proportion of total polar lipid radioactivity followed by phosphatidyl ethanolamine (PE). Phosphatidyl serine (PS) and sphingomyelin also exhibited significant differences in the amount of radioactivity retained. Table II shows the percent distribution of radioactivity in various fatty acids of the brain as determined by radio-GLC following injection of the two tracers. The identification of the fatty acids was confirmed by comparing retention times with authentic standards before and after hydrogenation. The most striking result was the absence of radioactive peaks for 20:4 and 22:6 following injection of glucose. A further confirmation of this difference was sought by first concentrating the fatty acids separated according to the degree of unsaturation by the $AgNO_3$:SiO_2 TLC method. Sufficient amount of each fraction (monoene, diene, etc.) was collected by repeated chromatography of the original fatty acid mixture. Each fraction was then reexamined by radio-GLC before and after reductive ozonolysis. Three mg of total fatty acids (18,384 cpm) were put on each $AgNO_3$:SiO_2 TLC plate; 3-4 such plates were used to separate the fatty acids by degree of unsaturation. The tetraene and hexaene fractions were collected and individually pooled. These were then used for radio-GLC studies. The unsaturated fatty acid fractions from the $AgNO_3$:SiO_2 TLC of glucose injected animals failed to give any radioactive peaks except that for 18:1ω9. All other fractions such as dienes, tetraenes, etc., gave no radioactive peaks although distinct mass peaks were observed. On the other hand, the fatty acid fractions from acetate injected animals containing about the same amount of initial radioactivity gave distinct radioactive peaks that were identified by ozonolysis which produced radioactive aldehyde-ester peaks. These were as follows: monoenes–18:1, 20:1, 22:1, and 24:1, all ω9 in structure; diene–20:2ω6; triene–20:3ω6; tetraenes–20:4 and 22:4, both ω6; and, hexaene–22:6ω3. The distribution of radioactivity in palmitic acid (% RCA[1]) was 11.8% in the carboxyl carbon as compared to the whole following [1-^{14}C]-acetate injection and 5.6% after [U-^{14}C]-glucose; both are close to theoretical value for de novo synthesis, considering that acetate produced from [U-^{14}C] glucose is labeled in both the carbons. In the case of stearate, the carboxyl carbon had 19.4% radioactivity after [1-^{14}C] acetate and 12.2% after [U-^{14}C]-glucose injection.

$$1\%\ \text{RCA} = \frac{\text{Rad. Act. in } -\text{COOH}}{\text{Rad. Act. in Fatty Acid}} \times 100.$$

DISCUSSION

Brain palmitate isolated after glucose injection contained a higher proportion of radioactivity as compared to that obtained after acetate administration. This indicated a rapid glycolysis and oxidation of the glucose to provide acetyl CoA for the de novo synthesis of palmitate. It could also be due to a slower rate of activation of the injected acetate to acetyl CoA. The differences in the proportion of radioactivity in 18:0 and 18:1 after the two tracers were relatively minor. Thus, the acetyl CoA, formed from either glucose or acetate, used for elongation of palmitate seems to be part of one homogeneous pool. However, the absence of radioactivity (even after reexamination following concentration of the fatty acids according to the degree of unsaturation) following [U-^{14}C]glucose in the polyunsaturated fatty acids (20:4 and 22:6) was surprising, particularly so when most of the known products of fatty acids formed by elongation and desaturation were clearly radiolabeled following [1-^{14}C]acetate injection. If the acetyl CoA pool were homogeneous, the acetyl CoA should have been available for elongation of both palmitate and endogenous 18:2 or 18:3 to form 20:4 and 22:6, respectively. The absence of such labeling from acetyl CoA produced by glycolysis suggests the possibility of a non-homogeneous pool. Such a possibility has been suggested by Fritz (11). Finally, another possibility cannot be overlooked; for example, the difference in the utilization of acetyl CoA could be due to the fact that the formation of acetate from pyruvate occurs in the mitochondria and so acetyl CoA is available mainly to the mitochondrial elongation system and this system, in the brain, readily elongates 16:0 to 18:0 (12).

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Sorbic Acid Containing Triglycerides in Aphids and Their Fractionation by High Pressure Liquid Chromatography

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ABSTRACT

The sorbic acid containing triglycerides found in various aphid species have been fractionated by high pressure liquid chromatography. Mass spectral analysis afforded identification of three isolates as 2-*trans, trans*-sorbo-1, 3 dimyristin; 2-*trans, trans*-sorbo-1,3 myristopalmitin; and 2-*trans, trans*-sorbo-1,3 dipalmitin. No evidence of asymmetry was found by circular dichroism. The composition and proportions of these triglycerides was found to be species variable.

INTRODUCTION

Several studies on the chemical composition of aphid constituents have established that the major triglycerides present in certain aphid species are sorbic acid containing and therefore UV absorbing triglycerides. Bowie and Cameron (1) employed a silicic acid column to isolate 2-*trans, trans*-sorbo-1, 3-dimyristin as 65% of the triglyceride fraction from the large bronze aphid *Dactynotus jaceae.* Brown et al. (2) found that the triglyceride mixture from the bright orange aphid *Aphis nerii* contained 2-*trans, trans*-sorbo-1,3-dipalmitin as its major component. Shimizu (3) observed that an ether extract of *Dactynotus rudbeckiae* showed a strongly UV absorbing spot just below the normal glyceride fraction. Upon hydrolysis, this spot contained myristic and sorbic acid in a 2:1 ratio, respectively. Shimizu (3) speculated that, since sorbic acid and its esters are known to be both fungicidal and yeast inhibiting, they may serve an analogous function as defensive compounds for aphids.

The present study sought to ascertain if there is aphid species differentiation in the composition and proportions of sorbic acid containing triglycerides and also to test the efficacy of high pressure liquid chromatography (HPLC) as a technique for the separation of such structurally similar triglycerides.

EXPERIMENTAL PROCEDURES

Aphids of different pigmentations from a variety of greenhouse or local host plants were collected in early summer, extracted whole in a small volume (1-3 ml) of cyclohexane, and the extracts (1-10 μl) fractionated by HPLC.

HPLC parameters	
Model:	DuPont 841
Column:	Zorbax Sil, L: 250 mm, ID: 2.10 mm
Temp.:	Ambient
Mobile phase:	CH_2Cl_2:cyclohexane 2:1, vv
Pressure:	2000 psig
Flow:	0.4 ml/min
Detector:	UV photometer @ 254 nm
Detector sensitivity:	32 x 10^{-2} absorbance units
Chart speed:	5 mm/min

Three fractions from an *Aphis nerii* extract (host plant *Asclepias incarnata*) corresponding to peaks of UV absorption were collected following their fractionation by HPLC. Each fraction was monitored for purity by rechromatography and analyzed by mass spectrometry. Mass spectra were taken with a DuPont 21-490B single focus mass spectrometer at 70 ev, source: 210 C, probe: 100 C.

The identities of the major UV absorbing triglycerides isolated from other aphid spp. were established by subsequent co-chromatography with the *Aphis nerii* extract.

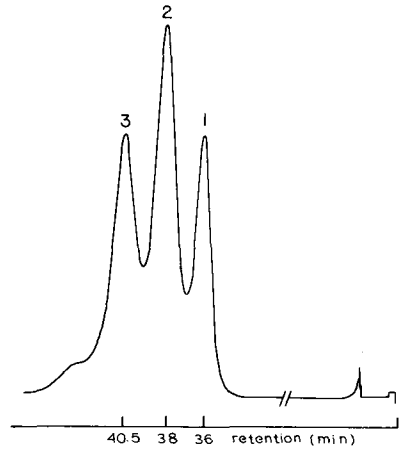

FIG. 1. High performance liquid chromatography elution pattern of three sorbic acid containing triglycerides from *Aphis neri* extract. Peak 1: 2-*trans, trans*-sorbo-1,3-dipalmitin; Peak 2: 2-*trans, trans*-sorbo-1,3-myristopalmitin; Peak 3: 2-*trans, trans*-sorbo-1,3-dimyristin. Host Plant: *Asclepias incaranta.* Detector sensitivity: 32 x 10^{-2} absorbance units.

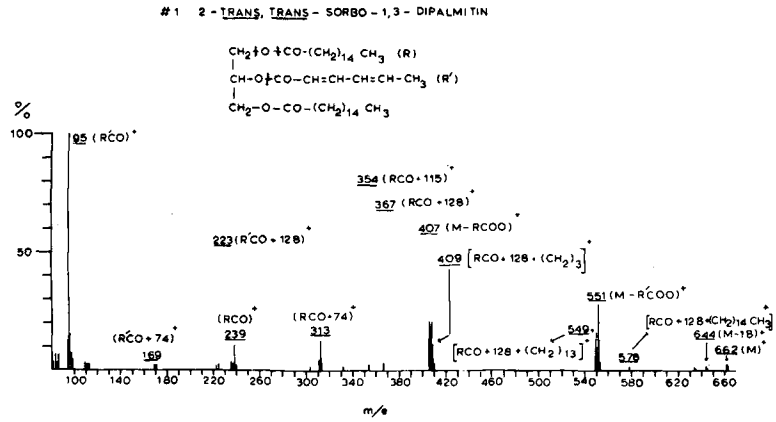

FIG. 2. Mass spectrum of UV absorption peak #1 from *Aphis neri* extract.

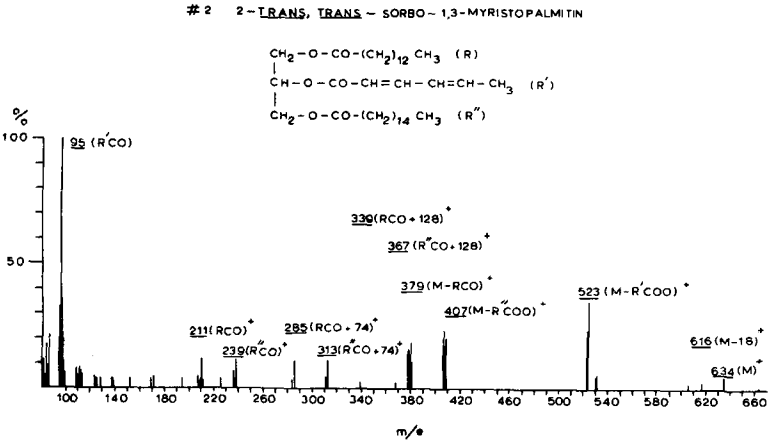

FIG. 3. Mass spectrum of UV absorption peak #2 from Aphis nerii extract.

No evidence of asymmetry in the isolates could be detected by circular dichroism analysis: (ϕ) in a range of 200-400 nm taken with a JASCO J40 instrument was zero.

RESULTS

HPLC of the *Aphis nerii* extract yielded three major UV absorbing peaks 1, 2, and 3 (Fig. 1). Mass spectral analysis of each of these components yielded spectra which correlated well with the major fragmentation patterns characteristic of triglycerides as reported by Barber et al. (4) and Lauer et al. (5).

Peak 1: 2-*trans*, *trans*-sorbo-1, 3-dipalmitin (Fig. 2) m/e 662 $[M]^+$, 644 $[M-18]^+$, 578 $[RCO + 128 + (CH_2)_{14}CH_3]^+$, 551 $[M-R'COO]^+$, 549 $[RCO + 128 + (CH_2)_{13}]^+$, 409 $[RCO + 128 (CH_2)_3]^+$, 407 $[M-RCOO]^+$, 367 $[RCO + 128]^+$, 354 $[RCO + 115]^+$ 313 $[RCO + 74]^+$, 239 $[RCO]^+$, 223 $[R'CO + 128]$, 169 $[R'CO + 74]^+$, 95 $[R'CO]^+$.

R: $-(CH_2)_{14}CH_3$
R': $-CH=CH-CH=CH-CH_3$

Peak 2: 2-*trans*, *trans*-sorbo-1,3-myristopalmitin (Fig. 3) m/e 634 $[M]^+$, 616 $[M-18]^+$ 523 $[M-R'COO]^+$, 407 $[M-R''COO]^+$, 379 $[M-RCO]^+$, 367 $[R''CO + 128]^+$ 339 $[RCO + 128]^+$, 313 $[R''CO + 74]^+$, 285 $[RCO + 74]^+$, 239 $[R''CO]^+$, 211 $[RCO]^+$, 95 $[R'CO]^+$.

R: $-(CH_2)_{12}CH_3$
R': $-CH=CH-CH=CH-CH_3$
R'': $-(CH_2)_{14}CH_3$

Peak 3: 2-*trans*, *trans*-sorbo-1, 3-dimyristin (Fig. 4) m/e 606 $[M]^+$, 588 $[M-18]^+$, 522 $[RCO + 128 + (CH_2)_{12}CH_3]^+$, 495 $[M-R'COO]^+$, 493 $[RCO + 128 + (CH_2)_{11}]^+$, 395 $[RCO + 128 + (CH_2)_4]^+$, 381 $[RCO + 128 + (CH_2)_3]^+$, 379 $[M-RCOO]^+$, 399 $[RCO +$

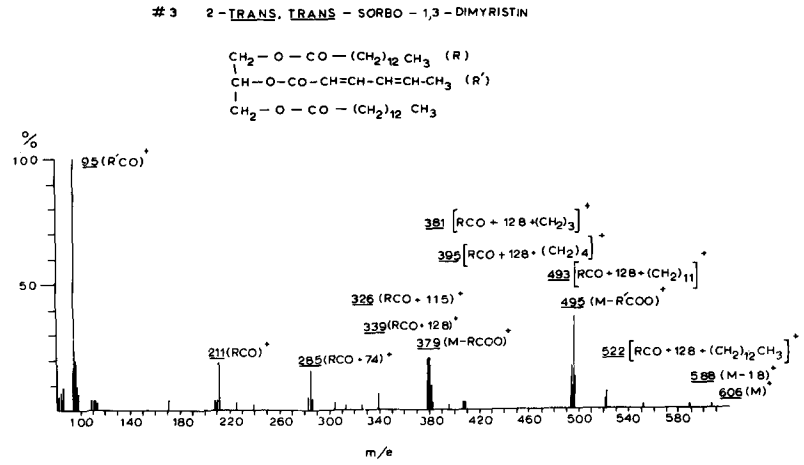

FIG. 4. Mass spectrum of UV absorption peak #3 from *Aphis nerii* extract.

TABLE I

Occurrence and Relative Proportions of Sorbic Acid Containing Triglycerides in Aphids

Aphid pigmentation/host	UV absorption peak				
	Unidentified	(1)[a]	(2)[b]	(3)[c]	Unidentified
Bright orange[d] / *Asclepias incarnata*		+	++	+	trace
Green[e] / *Rosa spp*				+	
Black / *Circium lanceolatum*				+	
Bright orange[d] / *Solidago spp*				+	trace
Bright orange[d] / *Lactuca saligna*				+	
Mixed / *D. stramonium*			trace	+	trace
Mixed / *Chrysanthemum spp*			trace	+	trace
None (white) / *Rubus occidentalis*	trace	+	++	+	
Green / *Spirea cantoniensis*			++	+	

[a] 2-*trans, trans*-sorbo-1,3-dipalmitin.
[b] 2-*trans, trans*-sorbo-1,3-myristopalmitin.
[c] 2-*trans, trans*-sorbo-1,3-dimyristin.
[d] *Aphis neri.*
[e] *Aphis rosa.*

128]$^+$, 326 [RCO + 115]$^+$ 285 [RCO + 74]$^+$, 211 [RCO]$^+$, 95 [R'CO]$^+$.

R: $-(CH_2)_{12}CH_3$
R': $-CH{=}CH-CH{=}CH-CH_3$

HPLC of the extracts from variously pigmented aphids hosted by nine host plants showed distinct species and host plant variation in both the content and proportions of sorbic acid containing triglycerides present (Table I). Since host plants are not the source of the sorbic acid in aphids (3), individual host plant conditions or constituents may influence the amounts and identities of those fatty acids which ultimately are esterified to yield the variety of sorbic acid containing triglycerides in aphids.

Assignments of peaks in these instances were based on their individual elution patterns and the peak enhancement observed when they were co-chromatographed simultaneously with the *Aphis nerii* extract.

DISCUSSION

Although the separation of triglycerides by various types of thin layer and column chromatography has been reported, these methods are generally effective mostly for the separation of triglycerides with differing degrees of saturation or for positional isomers. Triglycerides differing by one or two carbon atoms can be separated by gas liquid chromatography (6), but here, especially at higher temperatures, column bleeding becomes a serious problem with unsaturated triglycerides.

The most salient feature in the mass spectra

of the three isolates is the prominence of the base peak fragment, m/e 95, which is attributable to the sorbic acid acyl ion $[R'CO]^+$. The corresponding acyl ions for the palmitin and myristin residues at m/e 239 and 211, respectively, are 80-90% less abundant.

As was expected, all three spectra showed prominent peaks at m/e 94, assignable to the $[R'CO\text{-}1]^+$ ion which is characteristic of unsaturated triglycerides (5). The $[R'CO\text{-}1]^+/[R'CO]^+$ ratios for 1, 2, and 3 were 0.13, 0.33, and 0.16, respectively.

Assignment of the *sn*-positions of fatty acids in some triglycerides based on the loss of acyloxymethylene groups from the *sn*-1- and *sn*-3-positions has been reported (5,6). However, in the present work, recognizable $[M\text{-}RCOOCH_2]^+$ fragments were not observed for any position. The lack of these ions may reflect a different fragmentation pattern based on the extremely labile sorbic acid residue.

The *sn*-2-position assigned to sorbic acid supports the earlier observation of Hilditch and Williams (7) that in mixed saturated-unsaturated triglycerides of vegetable fats an unsaturated residue usually esterifies the *sn*-2-position.

The recent and remarkable developments in high performance liquid chromatography seem to hold promise for facile, rapid, and quantitative separation of minute amounts of triglycerides. However, the lack of specific UV absorption in normal triglycerides compels one to utilize the less sensitive refractive index or unreliable end absorption (e.g., 206 nm) for detection.

Due to their conveniently located absorption maxima (258 nm), the sorbic acid containing triglycerides found in aphids provided a unique opportunity to test the resolution and sensitivity of HPLC for triglyceride separation. The results are very promising. Triglycerides differing by only two methylene groups were easily separated by absorption chromatography on Zorbax Sil column.

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Nutritional Effects of Partially Hydrogenated Low Erucic Rapeseed Oils[1]

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ABSTRACT

The incidence of cardiac lesions in male rats fed rapeseed oil (*Brassica campestris*, cultivar 'Span') was lower with partially hydrogenated oil (iodine value 78) than with the liquid oil which had been treated in various ways. Another rapeseed oil (*Brassica napus*, cultivar 'Tower') was similarly improved when hydrogenated to iodine value 76.6, but not at iodine value 97.1, as demonstrated in both Sprague-Dawley and Wistar rats. The improved nutritional quality of hydrogenated oil appeared not to be related to the decreased concentration of linolenic acid, because that fatty acid in linseed oil with or without erucic acid did not increase the incidence of lesions. A relatively high concentration of docosahexaenoic acid in the cardiac fatty acids was observed in adversely affected groups, but a lower concentration was found with the appropriately hydrogenated rapeseed oil.

INTRODUCTION

Since a large portion of the vegetable oil consumed by man is partially hydrogenated, the nutritional properties of such food are of considerable interest.

Partial hydrogenation of a high erucic rapeseed oil was found to reduce the degree of cardiac lipidosis produced in the rat (1,2). The extent of the lipid deposition in rats receiving liquid rapeseed oil was related to the intake of erucic acid (2-5); the somewhat lesser changes in rats fed partially hydrogenated oil could be attributed to lower absorption of fats of higher melting point (6) and more specifically to that of *trans*-docosenoic acid compared to *cis*-docosenoic (7). The development of the longer term cardiac lesions of necrosis or fibrosis appeared to be unaffected by the partial hydrogenation of high docosenoic oil, fed as 15% by weight of the diet or 30% of calories (8). A different situation occurred after hydrogenation of low erucic rapeseed oil when a decreased incidence of cardiac lesions was observed (7-9). It has been postulated that linolenic acid, which exists in unhydrogenated rapeseed oil, plays a role in the etiology of cardiac lesions (10).

To ascertain the type of change which might be responsible for the improved nutritional quality of low erucic rapeseed oil that had been partially hydrogenated, the following experiments were undertaken.

MATERIALS AND METHODS

Span Rapeseed Oil

Oil from Span, a cultivar of *Brassica campestris*, known to produce cardiac lesions, was processed in different ways by Canada Packers Research and Development Laboratories, Toronto. One portion of the alkali refined, bleached, and deodorized Span oil was treated with carbon black and aluminium oxide to remove polar compounds. Another was treated as for hydrogenation in the presence of heat and nickel catalyst, but nitrogen instead of hydrogen was used. Another portion of oil was partially hydrogenated to an iodine value of 78.4.

Tower Rapeseed Oil

Oil from Tower, a cultivar of *Brassica napus*

[1]Presented in part at the AOCS Meeting, Chicago, September 1976.

TABLE I

Principal Fatty Acids of Control Fat (Lard and Corn Oil) and of Span Rapeseed Oil Before and After Various Treatments

	16:0	18:0	18:1	18:2	18:3	20:1	22:1	*trans*
Lard: Corn oil	22.7	12.5	37.9	21.5	0.1	0.6	---	---
Span oil	3.1	1.7	54.8	20.7	10.2	3.6	4.6	---
Span oil – Polar Compounds	3.2	1.6	54.7	20.8	10.1	3.8	4.7	---
Span oil + N_2	3.3	1.5	55.2	20.7	9.7	3.8	4.6	1.3
Span oil + H_2	3.3	5.4	79.3	2.8	1.4	3.0	3.8	46.7

TABLE II

Frequency of Cardiac Lesions in Sprague-Dawley Rats Fed Span Oils

	Cardiac lesions					
	Grade				Total	Percent
Dietary fat	1	2	3	4		
Lard: Corn oil	4	-	-	-	4/20	20
Span oil	3	5	3	6	17/20	85
Span oil – Polar compounds	1	3	3	-	7/10	70
Span oil + N_2	1	4	3	1	9/10	90
Span oil + H_2	3	-	-	-	3/10	30

TABLE III

Fatty Acid Composition of Unhydrogenated and Partially Hydrogenated Tower Oils

Fatty acid	Unhydrogenated	I.V.[a] 97.1	I.V. 76.6
16:0	4.3	4.3	4.6
16:1	0.2	0.4	0.2
17:1	0.1	0.1	0.1
18:0	2.0	2.9	11.7
18:1	59.3	68.7	72.4
18:2	20.7	14.9	5.8
18:3	8.1	1.7	0.5
20:0	1.1	1.3	0.7
20:1	2.1	3.8	2.0
22:0	0.5	0.4	0.4
22:1	1.4	1.3	1.5
24:1	0.2	0.2	0.1
Total PUFA	28.8	16.6	6.3
cis-PUFA[b]	25.2	7.3	0.3
trans-FA	0.8	13.6	29.9

[a]Iodine value.

[b]As determined by lipoxygenase. PUFA = polyunsaturated fatty acids.

which is low in erucic acid and glucosinolates, was alkali refined, bleached, and deodorized. Some Tower oil was partially hydrogenated to an iodine value of 97.1 and some to an iodine value of 76.6 by Canada Packers.

Other Vegetable Oils

Olive oil, sold commercially by Pastene, was used alone or with Target rapeseed oil containing 39.6% erucic to supply ca. 3% erucic acid in the final oil mixture. Linseed oil, made available by J.D. Jones, Food Research Institute, Agriculture Canada, was similarly tested with and without added rapeseed oil to give a mixture containing 3% erucic acid. A reference fat mixture of lard and corn oil (3:1) was fed to a group of rats in each experiment.

The test oils were fed as 20% by weight in a purified basal diet providing 25% casein, 30% cornstarch, 15% sucrose, 3% salt mixture (11), 1% vitamin mixture (2), and 6% alphacel. The diets were fed daily and ad libitum to Sprague-Dawley derived or Wistar rats obtained after weaning from Canadian Breeding Farms, St. Constant, Quebec.

Following 16 weeks of experimental feeding, the rats were fasted 4 to 5 hours, anesthetized with ether; the hearts were removed and those designated for histopathological examination were fixed in buffered formalin. The heart was then transversely cut into four portions, embedded in paraffin, and sections of 5 μ stained with hematoxylin-eosin. Histopathological examinations were done without knowledge of dietary treatments.

Cardiac lipids were extracted from the remaining hearts by the procedure of Bligh and Dyer (12,13) and the fatty acid composition determined by gas liquid chromatography (14) using both a Hewlett-Packard instrument, model 762A, equipped with a packed, butanediol succinate column and a Perkin Elmer, model 990, equipped with a Scott butanediol succinate column. *Trans* fatty acids were measured by IR spectrophotometry (15),

TABLE IV

Frequency of Cardiac Lesions in Sprague-Dawley and Wistar Rats Fed Unhydrogenated and Partially Hydrogenated Tower Oils in Two Time Replicates, A and B, for 16 Weeks

		Grade of cardiac lesion										
		0		1		2		3		4		
Rat strain	Dietary oil	A	B	A	B	A	B	A	B	A	B	Total lesions
Sprague-Dawley	Control	9	7	0	1	0	0	0	2	0	0	3/20
	Tower-U	4	3	1	2	2	3	3	1	0	1	13/20
	Tower 97	4	1	2	4	1	2	1	2	1	1	14/19
	Tower 77	6	5	2	1	1	3	1	1	0	0	9/20
Wistar	Control	10	10	0	0	0	0	0	0	0	0	0/20
	Tower-U	4	4	3	6	3	0	0	0	0	0	12/20
	Tower 97	7	3	3	4	0	3	0	0	0	0	10/20
	Tower 77	9	6	0	3	1	0	0	0	0	0	4/19

TABLE V

Cardiac Fatty Acids of Sprague-Dawley Rats Fed Tower Oils (% by wt)

Fatty acid	Lard + Corn oil	Tower-U	Tower-97	Tower-77
16:0	10.2 ± 0.5[a]	8.4 ± 0.8	8.4 ± 0.2	7.5 ± 0.4
16:1	0.6 ± 0.1	0.7 ± 0.1	1.1 ± 0.1	1.2 ± 0.2
18:0	21.5 ± 0.5	19.0 ± 1.0	15.1 ± 0.2	15.7 ± 0.7
18:1	10.4 ± 0.6	18.3 ± 2.3	22.9 ± 0.9	27.3 ± 2.9
18:2	15.0 ± 0.4	15.1 ± 1.2	12.7 ± 0.3	11.5 ± 1.2
18:3	0.6 ± 0.1	0.3 ± 0.2	0.2 ± 0.1	0.8 ± 0.2
20:1	0.5 ± 0.2	0.6 ± 0.2	0.5 ± 0.1	1.2 ± 0.2
20:2	0.3 ± 0.1	1.5 ± 1.1	0.3 ± 0.1	4.5 ± 1.0
20:3	0.3 ± 0.1	0.6 ± 0.3	0.3 ± 0.1	1.3 ± 0.3
20:4ω6	23.4 ± 0.7	20.1 ± 0.3	23.7 ± 0.5	17.7 ± 0.6
22:5ω6	5.4 ± 0.5	1.4 ± 0.3	0.6 ± 0.8	1.2 ± 0.3
22:5ω3	0.9 ± 0.1	1.2 ± 0.3	0.8 ± 0.3	0.4 ± 0.4
22:6ω3	4.5 ± 0.4	9.9 ± 1.4	9.3 ± 0.5	5.8 ± 1.3
20:4/22:6	5.2	2.0	2.5	3.1
22:5+6ω3	5.4	11.1	10.1	6.2
Total fatty acids				
mg/heart	29.9 ± 2.4	29.9 ± 2.3	27.1 ± 1.5	30.4 ± 1.8
mg/g heart	22.7 ± 1.8	21.8 ± 1.6	21.3 ± 0.8	23.1 ± 1.0

[a]Mean ± standard error of mean for five hearts.

and fatty acids containing the *cis*, *cis*-1,4-pentadienoic structure by lipoxidase (16).

RESULTS

The Span rapeseed oil contained under 4% eicosenoic acid, under 5% erucic acid, and as much octadecadienoic acid as the control fat mixture (Table I). The levels of these fatty acids were unchanged by either removal of polar compounds or treatment with nitrogen. During the heating of the oil with the nickel catalyst and nitrogen, a small amount of *cis* to *trans* isomerization had occurred. Partial hydrogenation of the Span oil had reduced the 18:2 and 18:3 to low levels and converted close to half of the fatty acids to the *trans* configuration, based on a standard of elaidic acid.

After these oils were fed to Sprague-Dawley rats for 16 weeks, cardiac necrosis or fibrosis was observed according to the distribution shown in Table II. The proportion of hearts with lesions was essentially unchanged by either removal of polar compounds or treatment with nitrogen. Only the partial hydrogenation of the oil reduced the severity and the occurrence of cardiac lesions.

The fatty acid compositions of the unhydrogenated and partially hydrogenated Tower oils are shown in Table III. A decrease in the polyenoic acids, particularly those containing *cis* double bonds, and an increase in *trans* double bonds were apparent with the successive degrees of hydrogenation. The Sprague-Dawley rats showed more background lesions and more severe lesions than the Wistar rats (Table IV). A relatively high incidence of cardiac lesions ap-

TABLE VI

Polyenoic Fatty Acids in the Heart of Wistar Rats Fed Tower Oils (% by wt)

Fatty acid	Lard + Corn oil	Tower-U	Tower-97	Tower-77
18:2ω6	14.1 ± 0.3[a]	15.2 ± 0.4	12.4 ± 0.5	9.5 ± 0.4
20:4ω6	24.1 ± 0.4	21.0 ± 0.5	23.5 ± 0.2	18.8 ± 0.4
22:5ω6	4.1 ± 0.6	tr	0.2 ± 0.2	1.1 ± 0.9
22:5ω3	0.6 ± 0.1	2.5 ± 0.3	0.3 ± 0.2	tr
22:6ω3	3.6 ± 0.3	10.0 ± 0.4	7.5 ± 0.3	4.5 ± 0.1
20:4/22:6	6.7	2.1	3.3	4.2
22:5+6ω3	4.2	12.5	7.8	4.5
Total fatty acids				
mg/heart	20.1 ± 1.5	19.2 ± 1.3	20.4 ± 1.5	18.2 ± 1.2
mg/g heart	15.4 ± 0.9	15.6 ± 0.7	15.1 ± 0.8	13.8 ± 1.0

[a]Mean ± standard error of mean for five hearts.

TABLE VII

Cardiac Lesions of Wistar Rats Fed Olive Oil High in Oleic Acid, Linseed Oil High in Linolenic Acid or Tower Oil

				Cardiac lesions		
				Grade		
Dietary fat	18:1	18:2	18:3	1	2	Total
Lard + Corn oil	38.0	21.6	0.1	1	0	1/15
Olive oil	72.1	11.6	0.9	1	0	1/15
Olive oil + 22:1	68.1	11.8	1.5	3	0	3/15
Linseed oil	13.21	17.1	61.1	2	0	2/15
Linseed oil + 22:1	13.5	16.8	57.3	4	0	4/15
Tower oil	44.3	29.0	13.6	4	2	6/15

[a]Total fat contains 3% 22:1 from Target rapeseed oil.

peared in both strains of rats fed either the unhydrogenated Tower oil or that oil hydrogenated to an iodine value of 97. The last group, fed the more hydrogenated oil, was not significantly different from the control group (17).

The cardiac fatty acids of the Sprague-Dawley rats fed the control mixture and the different Tower oils are shown in Table V. The concentration of octadecenoic acid increased with each stage of hydrogenation. The unhydrogenated oil and that having an iodine value of 97 were associated with a relatively high concentration of docosahexaenoic acid. This fatty acid showed the same retention time in eluting from the column for different experimental groups of hearts and was checked on two instruments. It, therefore, appeared that docosahexaenoic acid was mostly responsible for a high total concentration of ω3 fatty acids and for the low ratio of arachidonic acid to docosahexaenoic acid. This ω3 fatty acid in the hearts of rats fed the more hydrogenated oil (iodine value 77) approached the lower concentration of that found in the control animals.

The polyenoic fatty acids in the hearts of the Wistar rats (Table VI) showed similar characteristics to those found in Sprague-Dawley rats.

Olive oil high in oleic acid and linseed oil high in linolenic acid, with or without 3% erucic acid from rapeseed oil, did not significantly increase the incidence of cardiac lesions above the control level, but Tower oil did (Table VII). These Wistar rats exhibited low grade lesions with the 2 grade occurring only in rats fed low erucic rapeseed oil.

The high dietary level of linolenic acid increased the concentration of that acid and of docosapentaenoic acid in the cardiac lipids (Table VIII). Also, the concentration of cardiac octadecadienoic acid was higher with linseed oil than with Tower oil. Docosahexaenoic acid was significantly elevated in the cardiac fatty acids of all test groups compared to the control, and its high level in rats fed Tower oils did not differ from that in rats fed olive oil with some Target rapeseed oil. Linseed oil was not associated with the highest concentration of 22:6ω3.

TABLE VIII

Cardiac C_{20} and C_{22} Polyenoic Fatty Acids of Wistar Rats Fed Oils of Different Degrees of Unsaturation

Dietary fat	20:4ω6	22:4ω6	22:5ω3	22:6ω3	20:4/22:6	22:5+22:6
Lard + Corn oil	27.2 ± 1.6[a]	5.9 ± 0.7	0.7 ± 0.1	5.1 ± 0.4	5.3	5.8
Olive oil	25.4 ± 1.3	2.4 ± 0.2	0.5 ± 0.1	7.1 ± 0.4	3.6	7.6
Olive oil + 22:1[b]	24.7 ± 0.4	1.1 ± 0.1	0.6 ± 0.1	9.1 ± 0.1	2.7	9.7
Linseed oil	14.6 ± 1.1	—	4.0 ± 0.6	7.4 ± 1.0	2.0	11.4
Linseed oil + 22:1	15.4 ± 0.5	0.1 ± 0.1	5.3 ± 0.1	8.0 ± 0.4	1.9	13.3
Tower oil	20.3 ± 0.7	—	2.0 ± 0.3	10.3 ± 0.5	2.0	12.3

[a]Mean ± standard error of the mean for five hearts.
[b]Total fat contains 3% 22:1 from Tower rapeseed oil.

DISCUSSION

Hardening of low erucic rapeseed oils has been shown to improve their nutritional quality, as manifested by the reduction in the incidence of cardiac lesions. Since the partially hydrogenated oils were deficient in linoleic acid and yet improved the myocardial conditions, an essential fatty acid deficiency appears not to have been a responsible factor.

During partial hydrogenation a substantial portion of the double bonds was changed from a *cis* to a *trans* configuration, as has been found previously (18). Brassidic acid, the *trans* isomer of erucic acid, was found to be less available than the *cis* isomer (19), but was nevertheless incorporated into myocardial lipids (20-22). Whether or not the formation of docosenoic isomers in a low erucic rapeseed oil is an important factor in reducing cardiac lesions is not known. It must be remembered that hydrogenation may also produce important changes in the composition of minor components in the oil. At this stage, there is insufficient information to assess this possibility.

The increased level of docosahexaenoic acid in the cardiac lipids of rats from the most adversely affected groups indicated a change in membrane composition. It had been observed that the docosapolyenoic acids were largely confined to phosphatidyl choline and phosphatidyl ethanolamine of rat heart (23). Even without separation of the phospholipid fatty acids or the membranes, significant elevations in the level of docosahexaenoic acid were apparent in the total cardiac fatty acids in the rats fed liquid or mildly hydrogenated rapeseed oil.

Pokrovsky et al. (24) showed that mustard oil containing 34% erucic acid produced a significantly higher concentration of docosahexaenoic acid in liver microsomal lipids than did olive oil or a mixture of lard and sunflower oil. They proposed an index of the efficiency of essential fatty acid metabolism be obtained from the ratio of arachidonic acid to all other C_{20-22} polyenoic fatty acids. This combines ω3 and ω6 fatty acids without accounting for interrelationships of different families of fatty acids (25).

From experimental models of myocardial necrosis produced by either coronary occlusion or overstimulation with isoproterenol, Gudjarnason and Hallgrimsson (26) proposed that the development of the pathological condition was influenced by the relative amounts of polyenoic fatty acid in the cardiac membrane. If ω3 fatty acids replaced arachidonic acid, such substrates could not be used for prostaglandin synthesis and could disrupt cellular lipolytic activity and norepinephrine release. They demonstrated that 20:4/Σω3 decreased with age.

Linseed oil produced a high concentration of cardiac ω3-docosapentaenoic acid, derived from the dietary linolenic acid (25). The ω3-docosahexaenoic acid, presumably of the same origin, became more concentrated in the hearts of rats fed liquid or intermediately hydrogenated Tower oil than in those fed the other dietary fats, including linseed oil. Hydrogenation to the extent of eliminating conventionally detectable linolenic acid in Tower oil was also effective in reducing the level of cardiac docosahexaenoic acid to control levels. On the other side, a high dietary level of linolenic acid supplied by linseed oil appeared not to make the myocardium more susceptible to lesions. Nor did the addition of erucic acid to linseed oil alter the results. Only the *Brassica* oils themselves in these experiments were associated with an increase in both cardiac lesions and docosahexaenoic acid.

The level of docosahexaenoic acid appeared to be a better indicator of cardiac pathology than summations or ratios of polyenoic acids. A sufficient degree of hydrogenation was attained that both the incidence of cardiac lesions and the concentration of the membrane constitu-

ent, docosahexaenoic acid, approached those of reference controls.

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Acylglycerol Structure of Peanut Oils of Different Atherogenic Potential

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ABSTRACT

Detailed investigation was made of the triacylglycerol structure of native, simulated, and interesterified peanut oils, which had previously been shown to differ markedly in their atherogenic potential. By means of chromatographic and stereospecific analyses, it was shown that the more atherogenic native oil contains a significantly greater proportion of triacylglycerols with linoleic in *sn*-2-position and arachidic, behenic, and lignoceric acids in *sn*-3-position than the synthetic oils. It is suggested that the atherogenicity may arise from a relative metabolic unavailability of the linoleic acid from the native oil, which may be due in part to the presence of long chain saturated acids in the outer position. This might render the oil metabolically more saturated than the interesterified oils of the same total fatty acid composition, which contain a much greater proportion of the linoleic acid in the primary positions of the triacylglycerol molecule. The identification of specific triacylglycerols may allow the experimental testing of this hypothesis by feeding synthetic triacylglycerols incorporating the potentially atherogenic features.

INTRODUCTION

Extensive dietary trials have suggested that the triacylglycerol structure of a fat, as well as its fatty acid composition, is a determining factor in its atherogenic potency. Thus, native peanut oil has been shown (1) to be more atherogenic than corn oil when fed to rabbits in a diet containing 2% cholesterol and 6% fat. Randomized peanut oil and a simulated oil of the same fatty acid composition as peanut oil showed (2) an atherogenic potency indistinguishable from that of native corn oil, which was the least atherogenic of the seed oils that have been tested (3). It has also been observed that peanut oil, when included in a semipurified, cholesterol-free diet, is more atherogenic for rabbits than is corn oil. In this diet, however, peanut oil is less atherogenic than either coconut or butter oil (4).

The mechanism by which randomization of peanut oil leads to reduced atherogenicity is not clear, although a simple effect on cholesterol absorption would appear to be excluded (2). Since intact triacylglycerols are not absorbed, the atherogenic action of the oil must be exercised at the level of the acylglycerol intermediates of either the catabolic or anabolic processes of triacylglycerol metabolism. Since the enzymes involved in the triacylglycerol digestion, absorption, and eventual clearance from the blood stream are specific for the *sn*-1-position (5,6) or for both *sn*-1- and *sn*-3-positions (7), the nature and availability of the monoacylglycerol and diacylglycerol intermediates could be influenced by the composition, positional distribution, and molecular association of the fatty acids in the original triacylglycerol molecules.

The present study compares the acylglycerol structures of native, rearranged, and simulated peanut oils as revealed by thin layer (TLC) and gas liquid chromatography (GLC), and stereospecific analyses. The results have permitted the identification of several characteristic triacylglycerol structures which can be submitted to a dietary testing of their atherogenic potential.

MATERIALS AND METHODS

The various natural and experimental oils were as described by Kritchevsky et al. (2). Native peanut oil (PNO) was subjected to autointeresterification to yield the randomized peanut oil (PNOR) with fatty acid spectrum identical to that of the starting material. The special fat (PGF) was prepared by mixing olive oil (55%), cottonseed oil (10%), and safflower oil (35%). Arachidic and behenic acids were integrated into this fat (PGFR) by randomization (interesterification) in the presence of base. Native corn oil (CO) served as a reference fat. Synthetic, 1,2-, 2,3-, and 1,3-diacyl-*sn*-glycerols of the common saturated and unsaturated fatty acids were gifts from Dr. D. Buchnea.

Grignard Degradation of Triacylglycerols

The reaction was carried out as described by Yurkowski and Brockerhoff (8) but was scaled

TABLE I

Positional Distribution of Fatty Acids in Native and Interesterified Peanut and Corn Oils[a]

Fatty acids	PNO				PNOR	PGFR
	Pos. 1	Pos. 2	Pos. 3	Reconst.	Total	Total	Total
	Moles %						
16:0	19.9	2.2	12.8	11.6	11.6	11.5	10.5
16:1	0.06	0.07	0.2	0.1	0.1	0.1	0.56
18:0	3.5	1.0	3.7	2.7	2.8	2.7	2.2
18:1	47.5	50.8	54.0	50.8	50.8	50.9	42.3
18:2	26.1	46.2	11.3	27.9	27.8	27.9	39.1
18:3	–	–	–	–	–	–	0.34
20:0	0.3	–	4.0	1.6	1.4	1.4	1.3
20:1	0.7	–	2.9	1.2	1.4	1.3	0.34
22:0	0.1	0.1	7.3	2.5	2.6	2.6	3.0
24:0	–	–	3.3	1.1	1.1	1.1	0.32

Fatty acids	PGF				CO			
	Pos. 1	Pos. 2	Pos. 3	Total	Pos. 1	Pos. 2	Pos. 3	Total
14:0	0.3	0.2	0.0	0.2				
16:0	21.9	2.4	15.2	13.2	26.6	4.9	10.1	13.5
16:1	0.9	0.5	0.7	0.7	0.2	0.4	1.0	0.5
18:0	3.9	0.6	3.4	2.5	3.5	1.3	0.9	1.8
18:1	39.7	50.3	44.9	45.0	23.6	24.4	28.2	25.7
18:2	32.5	44.7	33.5	37.0	44.5	67.6	57.4	56.7
20:0	tr	tr	0.8	0.3	0.3	–	0.8	0.3
20:1	0.6	0.4	0.6	0.5	0.7	0.4	1.2	0.8
22:0	0.2	0.6	0.8	0.5	0.4	0.3	0.3	0.3
20:3	tr	0.3	0.1	0.1	0.4	0.7	0.1	0.4

[a]Abbreviations: PNO = native peanut oil; PNOR = interesterified peanut oil, PGF = mixture of cottonseed (10%), olive (55%), and safflower (35%) oils calculated to resemble peanut oil minus the long chain fatty acids (20:0; 22:0; 24:0); PGFR = PGF interesterified with arachidic and behenic glycerides synthesized to resemble PNO; CO = corn oil.

down for smaller quantities of triacylglycerol. The triacylglycerol (0-10 mg) dissolved in 0.4 ml dry diethyl ether was treated with 0.1 ml of 1 M ethyl-magnesium bromide in diethyl ether. After 25 sec, 0.1 ml of acetic acid-diethyl ether (1:9, v/v) was added, followed 30 sec later by 0.1 ml water. Subsequent to the addition of 4 ml of diethyl ether, the extract was washed with water, 2% sodium bicarbonate, and finally with water. The ether solution was dried over sodium sulfate and then evaporated under a stream of nitrogen. The 1,2(2,3)-diacylglycerols resulting from Grignard hydrolysis were isolated by TLC using borate-impregnated Silica Gel G and chloroform-acetone (96:4) as developing solvent (9).

Pancreatic Lipolysis

The enzyme digestion was performed essentially as described by Breckenridge and Kuksis (10). The reaction mixture was hand-shaken in a separatory funnel at room temperature for 10 min. The neutral lipids were then extracted with diethyl ether without acidification of the reaction mixture. The diacylglycerols were recovered by TLC as described above.

Stereospecific Analyses

The stereospecific analyses of the total oils and their subfractions isolated by $AgNO_3$-TLC were performed according to Brockerhoff (11). The mixed 1,2(2,3)-diacylglycerols were converted to the phenylphosphatides and purified by TLC. The phosphatides were then digested to completion with phospholipase A_2. The resulting fatty acids, lysophenylphosphatides and residual phenylphosphatides were separated by TLC and the fatty acid composition determined by GLC. The positional distribution of the fatty acids was calculated according to the methods outlined by Brockerhoff (12).

Thin Layer Chromatography

The triacylglycerols were purified by TLC on Silica Gel H (Merck and Co.) using heptane-isopropyl ether-acetic acid (60:40:4, v/v/v) as developing solvent. The purified triacylglycerols were separated by argentation TLC, using Silica Gel G impregnated with 10% $AgNO_3$ and benzene-diethyl ether (80:20, v/v) as develop-

ing solvent (13). Any incompletely resolved oligoene bands were eluted and rechromatographed, using benzene-diethyl ether (95:5). The amounts of the triacylglycerols in each subfraction were estimated by GLC of the triacylglycerols using tridecanoin as internal standard.

Gas Liquid Chromatography

Intact triacylglycerols and diacylglycerol TMS ethers were resolved according to molecular weight by GLC on 3% OV-1 (14) while intact diacylglycerols as the TMS ethers were resolved according to molecular weight and degree of unsaturation by GLC on SILAR 5CP (15) columns. Fatty acid methyl esters were analyzed on columns prepared with 10% EGSS-X as previously described (16). The fatty acid methyl esters were obtained, using 6% sulfuric acid in sbsolute methanol.

RESULTS

Analyses of Total Triacylglycerols

Table I gives the composition and positional distribution of fatty acids in the native and experimental oils. The results for the total fatty acids and the fatty acids in the 2-position of the natural oils agree well with previous analyses (2). The 2-position of peanut oil contains more linoleic acid than either the 1- or the 3-position. Linoleic acid is the major component of 2-fatty acids only in corn oil. The linoleic-oleic acid ratios in the 2-position of PNO, PGF, and CO are 0.91, 0.89, and 2.77, respectively.

The present data reveal that the acids in the *sn*-1- and *sn*-3-positions of the native peanut oil are markedly different. It contains about 50% more palmitic acid in position 1 than in position 3, both of which contain some 6 to 8 times as much as position 2. Although linoleic acid is also found in position 1 in nearly double the proportion in which it is found in position 3, position 2 contains 2 to 4 times as much as either of the two outer positions of the glycerol molecule. The long chain fatty acids, arachidic, behenic, and lignoceric acids are confined almost exclusively to position 3, while oleic acid is distributed nearly evenly among the three positions.

The positional distribution of the fatty acids in CO and PGF is nonrandom; both samples contain 1.5-2 times as much palmitic acid in position 1 as in position 3. Oleic acid is distributed almost equally among the three positions of PGF and CO, but minor dissimilarities may be noted. The largest deviations from randomness are seen in PGF, which has 21% less in 1- and 11% less in 3- than in 2-position. For CO, positions 1 and 2 are equal but position 3 has 16% more oleic acid. The smaller amounts of linoleic acid found in either of the outer positions of the glycerol molecule in these fats are distributed differently than in peanut oil. While PGF exhibits equal levels of linoleic acid in 1- and 3-, CO contains over 20% more than 3- than in 1-position. As expected, the stereospecific analyses of the randomized oils, PNOR and PGFR, showed that each fatty acid was present in each position at 33% of its concentration in the total oil. As a result, the partial acylglycerol intermediates generated by the various lipases and acyltransferases would be expected to differ markedly in fatty acid composition, positional distribution, and molecular association.

Table II gives the molecular weight distribution of the triacylglycerols in the native and synthetic oils and in the subfractions derived from them by argentation TLC. The differences in the molecular weight profiles among the subfractions are due to the differences in the composition and molecular association of the fatty acids in the individual triacylglycerol molecules. Table II also indicates marked differences in the proportions of the various triacylglycerol subfractions among the natural, rearranged, and synthetic peanut oils. This is due to a change in the molecular association of the fatty acids from a specific to a random placement, as shown below.

Table III gives the fatty acid composition of the various subfractions of peanut oil derived by argentation TLC. It is seen that the long chain fatty acids (arachidic, behenic, and lignoceric) are distributed about equally among the dienes, trienes, and tetraenes, as is palmitic acid. A significant amount of palmitate, however, is found in the triacylglycerols containing two saturated fatty acids per molecule. There are significant amounts of trioleoyl and trilinoleoylglycerols present in both the native and the interesterified peanut oils accounting for 30% of the trienes and 100% of the hexaenes, respectively.

On the basis of the stereospecific analyses of the total oils (Table I) and the fatty acid composition of the individual silver nitrate fractions (Table III), it is possible to identify the structures of the triacylglycerols containing the long chain saturated fatty acids. Thus, the tetraenes containing one saturated fatty acid are made up of *sn*-1, 2-dilinoleoylglycerols esterified at the *sn*-3-position with arachidic, behenic, and lignoceric acids. Likewise, the trienes with one saturated acid contain about 8% *sn*-1,2-oleoyllinoleoylglycerols esterified to the C_{20} to C_{24} saturated acids at the *sn*-3-position. The dienes

TABLE II

Molecular Weight Distribution of Triacylglycerols in Native and Interesterified Peanut Oils and in the Subfractions Derived from Them by Argentation Thin Layer Chromatography[a]

Carbon number	Oil			Carbon number	Oil		
	PNO	PNOR	PGFR[b]		PNO	PNOR	PGFR[b]
	Moles %						
Total	100	100	100	Trienes (111)	11.7	12.4	8.6
48	0.9	0.1	–	52	1.0	0.5	3.7
50	2.1	2.2	4.6	54	90.5	92.3	93.9
52	26.5	24.8	24.9	56	8.0	6.8	2.3
54	55.1	57.3	56.1	58	0.5	0.4	–
56	6.0	5.5	6.9	Trienes (012)	21.1	19.4	18.6
58	6.9	5.8	7.5	50		1.0	0.8
60	2.3	2.0	–	52	61.4	62.2	59.3
62		0.3	–	54	20.9	19.5	13.0
Monoenes (001)	5.4	5.8	4.1	56	6.6	6.9	7.5
48	0.6	–	0.5	58	9.2	8.8	17.3
50	27.2	34.6	36.0	60	1.9	1.5	2.0
52	17.0	17.1	15.4	Tetraenes (112)	21.1	23.0	23.2
54	13.5	10.8	10.6	52	1.4	0.8	2.5
56	24.4	17.9	22.9	54	98.6	99.2	95.9
58	13.8	11.2	7.4	Tetraenes (022)	7.3	5.0	8.6
60	3.5	3.7	3.1	52	47.8	56.6	60.5
Dienes (011)	16.4	15.8	10.3	54	13.6	19.1	12.7
50	1.2	0.2	1.5	56	7.6	6.2	7.3
52	47.5	56.5	58.3	58	21.1	12.8	17.6
54	20.5	16.2	13.3	60	9.9	4.8	1.9
56	9.3	7.6	7.6	Pentaenes (122)	12.1	12.1	21.4
58	15.2	13.1	17.1	52	1.7	1.3	1.7
60	5.8	6.1	2.1	54	98.3	98.7	97.6
Dienes (002)	2.5	3.1	3.7	Hexaenes (222)	1.6	1.6	6.7
50	35.5	35.5	36.6	54	100.0	100.0	99.9
52	18.3	16.7	15.3				
54	12.7	10.6	10.4				
56	21.6	18.1	23.2				
58	9.9	11.1	7.3				
60	1.8	3.5	3.0				
62		2.6	3.4				

[a]Abbreviations: PNO = native peanut oil; PNOR = interesterified peanut oil; PGF = mixture of cottonseed (10%), olive (55%), and safflower (35%) oils calculated to resemble peanut oil minus the long chain fatty acids (20:0; 22:0; 24:0); PGFR = PGF interesterified with arachidic and behenic glycerides synthesized to resemble PNO; CO = corn oil.

[b]Calculated on the basis of 1,2,3-random distribution.

containing two saturated acids are seen to contain about 14% *sn*-1,2-palmitoyl linoleoylglycerols esterified at the *sn*-3-position with the long chain saturated fatty acids. These estimates are consistent with the carbon number distributions of the triacylglycerols in the corresponding silver nitrate fractions (Table II).

A more complete assessment of the positional distribution of the fatty acids in the triaclyglycerol molecules is obtained by stereospecific analysis of the individual triacylglycerol fractions isolated by argentation TLC. Table IV gives the results of stereospecific analyses of the dienes containing one saturated acid and of the tetraenes containing two oleic acids and one linoleic acid. It is seen that these dienes are made up of two oleic acid residues in combination with one molecule of either palmitic in the *sn*-1- and *sn*-3-positions or one molecule of the C_{20} -C_{24} saturated acids in the *sn*-3-position. The tetraenes contained significantly more of the acylglycerols with oleic acid in the *sn*-3-position than in the *sn*-1-position. About 50% of these triacylglycerols contained linoleic acid in the 2-position.

Analyses of Diacylglycerols

Further information about the positional distribution and molecular association of the fatty acids in the triacylglycerol molecules was

TABLE III

Fatty Acid Composition of Various Subfractions of Native and Interesterified Peanut Oil Derived by Argentation Thin Layer Chromatograph[a]

	Subfractions of triacylglycerols[b]										
Fatty acids	001	011	002	111	012	112	022	122	222	Reconst.	Total
					Moles %						
PNO											
16:0	38.5	16.6	44.5		22.1	0.8	19.5	1.3		12.5	11.6
16:1		0.2		0.2		0.1				0.1	0.1
18:0	10.8	5.9	8.9		5.5	0.3	5.1	0.3		3.4	2.8
18:1	36.7	64.9		98.1	34.8	65.8	2.1	32.4	1.0	49.6	50.8
18:2			31.8		28.6	32.3	60.7	64.0	99.0	27.6	27.8
20:0	3.6	3.1	4.1		1.5		2.2			1.3	1.4
20:1	0.3	1.1		1.7	1.0	1.4		2.0		1.2	1.4
22:0	6.9	5.5	8.3		5.1		7.0			3.1	2.6
22:1				0.2							0.1
24:0	3.1	2.2	2.4		1.4		3.3			1.1	1.1
PNOR											
16:0		23.8			22.5	0.5	21.0	1.3		11.5	11.5
16:1		0.2		0.1		0.1				0.1	0.1
18:0		5.7			6.0	0.3	5.9	0.3		3.0	2.7
18:1		57.0		97.7	34.2	65.0	1.9	32.4	1.0	51.8	50.9
18:2		1.2			28.5	33.3	60.5	64.0	99.0	25.8	27.9
20:0		2.7			1.7		2.0			1.1	1.4
20:1		2.0		2.1	1.2	0.8		2.0		1.3	1.3
22:0		4.8			4.6		5.6			2.4	2.6
22:1				0.1							0.1
24:0		2.4			1.4		3.1			1.0	1.1

[a]Abbreviations: PNO = native peanut oil; PNOR = interesterified peanut oil, PGF = mixture of cottonseed (10%), olive (55%), and safflower (35%) oils calculated to resemble peanut oil minus the long chain fatty acids (20:0; 22:0; 24:0); PGFR = PGF interesterified with arachidic and behenic glycerides synthesized to resemble PNO; CO = corn oil.

[b]Bands 001, 011, and 002 of PNOR were pooled due to incomplete separation. The equivalent bands in PNO were resolved by rechromatographing on $AgNO_3$-TLC.

TABLE IV

Positional Distribution of Fatty Acids in Major Mixed Acid Triacylglycerol Subfractions of Native Peanut Oil Derived by Argentation Thin Layer Chromatography

	Sn-glycerol positions				
Fatty acids	1-	2-	3-	Total	Reconstituted
			Moles %		
011					
16:0	25.3	4.0	20.0	16.6	16.4
16:1	0.1	0.2	0.3	0.2	0.2
18:0	8.1	1.5	7.9	5.9	5.8
18:1	65.9	93.0	37.3	64.9	65.4
20:0	0.1	0.2	8.7	3.1	3.0
20:1	0.2	0.1	3.0	1.1	1.1
22:0	0.2	0.8	15.4	5.5	5.5
22:1				0.3	
24:0	0.1	0.3	6.7	2.2	2.4
112					
18:1	69.2	47.0	87.8	66.4	68.0
18:2	30.8	53.0	12.2	33.7	32.0

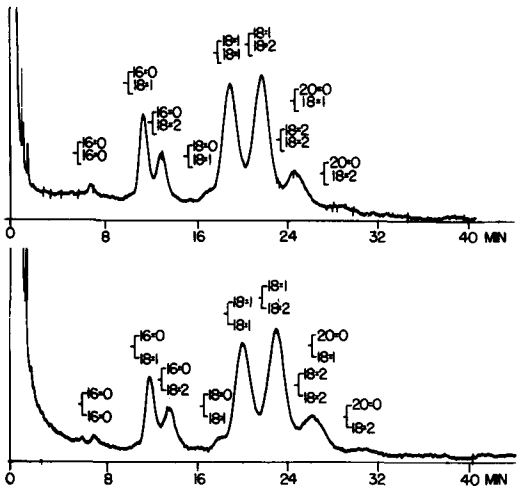

FIG. 1. Gas liquid chromaotgraphy (GLC) elution patterns of 1,2(2,3)- (upper) and 1,3- (lower) diacylglycerols of interesterified peanut oil. Peaks are identified by the pairing of fatty acids only, epimers and reversed isomers are not differentiated. GLC conditions as described by Myher and Kuksis (15).

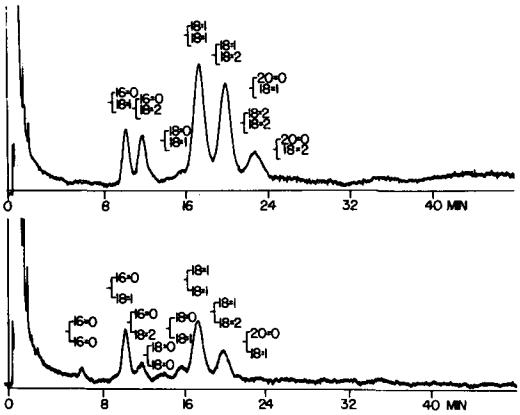

FIG. 2. Gas liquid chromatography (GLC) elution patterns of 1,2(2,3)- (upper) and 1,3- (lower) diacylglycerols of native peanut oil. Peaks are identified by the pairing of fatty acids only, epimers and reversed isomers are not differentiated. GLC conditions as in Figure 1.

obtained by a chromatographic examination of the diacylglycerols derived from the native and rearranged peanut oils by Grignard degradation. Figure 1 compares the gas liquid chromatographic elution patterns recorded for the 1,2(2,3)- and 1,3-diacylglycerols of interesterified peanut oil. Both of the elution patterns are identical as would have been anticipated if the fatty acid distributions among the three positions of the triacylglycerol molecule were truly random. Furthermore, the proportions of the various diacylglycerol species found agreed closely with the values calculated for a random cleavage of the triacylglycerol types calculated for the interesterified fat on the basis of a random distribution of fatty acids as shown in Table V. Figure 2 shows a similar comparison of the elution patterns for the 1,2(2,3)- and 1,3-diacylglycerols derived from the native peanut oil. The two gas liquid chromatograms are markedly different from each other and from those recorded for the diacylglycerols of the interesterified peanut oil. Again, the composition of the molecular species of the mixed 1,2(2,3)- and 1,3-diacylglycerols as determined by gas chromatography is closely similar to that calculated for the diacylglycerols derived from the native peanut oil by Grignard degradation and the knowledge of the positional distribution of fatty acids, as shown by the data in Table V.

Table V compares the composition of the molecular species of the *sn*-1,2-, *sn*-2,3-, mixed 1,2(2,3)- and X-1,3-diacylglycerols of the native and rearranged peanut oils as obtained by calculation and by gas liquid chromatographic resolution according to molecular weight and degree of unsaturation. The compositions of the *sn*-1,2- and *sn*-2,3-diacylglycerols in the native oil were calculated on the assumption of *sn*-1-random, *sn*-2-random and *sn*-2-random, *sn*-3-random combinations of the fatty acids in the corresponding positions of the triacylglycerols as given in Table I. These distributions indicate the pairings of the major fatty acids and reflect the nature of the enantiomeric native peanut oil. Addition and normalization of the estimates for the corresponding molecular species of the *sn*-1,2- and *sn*-2,3-diacylglycerols gives the calculated *sn*-1,2(2,3)-diacylglycerol composition, which may then be compared to the composition determined experimentally for the mixed *sn*-1,3-(2,3)-diacylglycerols by GLC on the SILAR 5CP and OV-1 columns. An inspection of the appropriate values (columns 3, 4, and 5 under PNO in Table V) reveals relatively close agreement, which suggests that the *sn*-1-random, *sn*-2-random and *sn*-2-random, *sn*-3-random calculation may provide a valid estimation of the pairing of fatty acids in the diacylglycerols. It should be noted, however, that the higher molecular weight diacylglycerols were not determined by the SILAR 5CP columns due to their low concentrations. A quantitative estimate of the high molecular weight diacylglycerols was obtained by GLC with the OV-1 column. Furthermore, in these comparisons, no account is taken of the reverse isomers of diacylglycerols.

Likewise the calculated composition of the X-1,3-diacylglycerols of the native peanut oil

TABLE V

Composition of Molecular Species of Diacylglycerols as Derived by Grignard Degradation of Native, Rearranged, and Synthetic Peanut Oils and by Calculation from Stereospecific Analyses[a]

Diacylglycerols[b]		PNO					PNO			PNOR		
		sn-1,2-	*sn*-2,3-	*sn*-1,2(2,3)-			x-1,3-			1,2(2,3); (1,3)-		
		Calc.	Calc.	Calc.	SILAR	OV-1	Calc.	SILAR	OV-1	Calc.	SILAR	OV-1
						Moles %						
16:0	16:0	0.44	0.28	0.36			2.55	2.0	1.4	1.32	0.7	0.9
16:0	16:1									0.02		
16:0	18:0	0.28	0.21	0.25			1.18	1.5		0.62	0.7	
16:0	18:1	11.15	7.69	9.42	8.9		16.82	15.9		11.71	9.8	
16:1	18:1	0.06	0.10	0.08			0.12			0.10		
16:0	18:2	9.77	6.16	7.97	8.7	16.2	5.59	6.3	21.2	6.42	6.5	17.6
16:1	18:2	0.03	0.09	0.06			0.06			0.10		
18:0	18:0						0.13					
16:0	20:0		0.09	0.05			0.80			0.32		
16:0	20:1		0.06	0.03	2.7		1.02	6.7		0.30		
18:0	18:1	2.25	2.42	2.34			3.65			2.75	2.8	
18:1	18:1	24.13	27.43	25.78	24.2	71.2	25.65	29.5	56.5	25.91	24.6	68.3
18:1	18:2	35.20	30.69	32.94	31.2		19.46	17.2		28.40	31.8	
18:2	18:2	12.06	5.22	8.64	10.9		2.95			7.78	11.1	
Other C_{36}				1.88			1.0			1.6		
16:0	22:0		0.16	0.08			1.45			0.60		
18:0	20:0		0.03	0.01			0.14			0.08		
18:1	20:0	0.15	2.06	1.11	ND[c]	6.0	2.06	ND	8.9	1.42	ND	6.1
18:1	20:1	0.36	1.47	0.92			1.76			1.32		
18:2	20:0	0.14	1.85	1.00			1.04			0.78		
18:2	20:1	0.32	1.34	0.83			0.83			0.73		
16:0	24:0		0.07	0.03			0.66			0.25		
18:1	22:0	0.06	3.76	1.91	ND	4.6	3.52	ND	8.8	2.65	ND	5.0
18:2	22:0		3.37	1.69			1.91			1.45		
18:1	24:0		1.67	0.84			1.57			1.12		
18:2	24:0		1.52	0.76	ND	2.0	0.86	ND	3.2	0.61	ND	1.9

[a]Abbreviations: PNO = native peanut oil; PNOR = interesterified peanut oil, PGF = mixture of cottonseed (10%), olive (55%), and safflower (35%) oils calculated to resemble peanut oil minus the long chain fatty acids (20:0; 22:0; 24:0); PGFR = PGF interesterified with arachidic and behenic glycerides synthesized to resemble PNO; CO = corn oil.

[b]C_{32}, C_{34}, and C_{36} from SILAR, 5CP, but sum normalized to OV-1 sum for these molecular weights. C_{38}, C_{40}, and C_{42} totals from OV-1 profiles.

[c]ND = not determined.

was obtained by assuming 1-random, 3-random combinations of the corresponding fatty acids as given in Table I. A comparison of the calculated composition of the X-1,3-diacylglycerols to that determined experimentally also shows good agreement either on the basis of molecular weight or degree of unsaturation or both. It may, therefore, be concluded that the 1-random, 3-random calculation gives an essentially correct estimation of the pairing of the fatty acids in the major diacylglycerols. This comparison does not distinguish, of course, between the enantiomers.

In the rearranged oil, all the diacylglycerol types possessed the same random composition of fatty acids in all positions. Furthermore, the calculated diacylglycerol compositions of this oil agreed closely with those determined experimentally as already noted in discussing Figures 1 and 2. These analyses indicate that the gas liquid chromatographic methods of analyses are sound and suitable for the assessment of the structure of the molecular species of the original triacylglycerols in the native and synthetic peanut oils.

TABLE VI

Composition of Molecular Species of Triacylglycerols of Native, Rearranged, and Synthetic Peanut Oils as Derived from Exhaustive Chromatographic Resolution and Stereospecific Analysis[a]

Rac-triacylglycerols			Reconstituted[b]			Calculated[c]		
			PNO	PNOR	PGFR	PNO	PNOR	PGFR
			Moles %					
16:0	16:0	16:0	–	0.1	0.08	0.06	0.15	0.12
16:0	18:0	16:0	–	0.02	0.01	0.02	0.04	0.03
16:0	16:0	18:0	–	0.07	0.05	0.03	0.07	0.05
16:0	18:0	18:0	–	0.01	0.01	–	–	–
16:0	16:0	20:0				0.02	0.04	0.01
16:0	16:0	22:0				0.03	0.07	0.04
16:0	16:0	24:0				0.01	0.03	–
16:0	18:1	16:0	1.02	0.47	0.32	1.29	0.67	0.49
16:0	18:1	18:0	0.76	0.31	0.21	0.60	0.32	0.20
16:0	16:0	18:1		1.17	1.15	0.37	1.35	0.98
16:0	18:0	18:1		0 18	0.17	0.17	0.32	0.20
18:0	16:0	18:1		0.19	0.12	0.04	0.32	0.20
18:0	18:0	18:1		0.03	0.02	0.02	0.04	0.04
16:0	16:0	20:1				0.01	0.03	–
16:0	18:1	20:0				0.40	0.16	0.12
16:0	18:1	22:0				0.74	0.30	0.28
16:0	18:1	24:0				0.33	0.13	0.03
18:1	20:0	16:0				–	0.16	0.12
18:1	22:0	16:0				–	0.30	0.28
18:1	24:0	16:0				–	0.13	0.03
16:0	18:2	16:0	0.98	0.28	0.28	1.18	0.37	0.45
16:0	18:2	18:0	0.72	0.17	0.19	0.55	0.17	0.19
16:0	18:1	18:1	8.01	5.30	4.52	8.55	5.96	3.96
16:0	16:0	18:2	–	0.78	0.98	0.12	0.74	0.90
16:0	18:0	18:2	–	0.11	0.15	0.03	0.17	0.19
18:0	18:1	18:1	2.3	0.88	0.49	1.85	1.40	0.83
18:0	16:0	18:2	–	0.11	0.11	0.03	0.17	0.19
18:0	18:0	18:2	–	0.02	0.02	0.01	0.04	0.04
18:1	18:0	18:1	–	0.43	0.34	0.26	0.70	0.42
18:1	16:0	18:1	–	2.91	2.26	0.56	2.98	1.96
16:0	18:1	20:1				0.29	0.15	0.03
16:0	20:1	18:1					0.15	0.03
16:0	18:2	20:0				0.37	0.09	0.11
16:0	18:2	22:0				0.67	0.17	0.26
16:0	18:2	24:0				0.30	0.07	0.03
18:1	18:1	20:0				1.05	0.72	0.48
18:1	18:1	22:0				1.79	1.35	1.15
18:1	18:1	24:0				0.80	0.57	0.13
18:1	20:0	18:1				0.01	0.36	0.48
18:1	22:0	18:1				0.02	0.67	1.15
18:1	24:0	18:1				0.01	0.28	0.13

TABLE VI (Contd.)

Rac-triacylglycerols			Reconstituted[b]			Calculated[c]		
			PNO	PNOR	PGFR	PNO	PNOR	PGFR
			Moles %					
16:0	18:2	18:1	7.62	3.20	3.98	7.77	3.27	3.66
16:0	18:1	18:2	2.96	3.18	3.90	2.84	3.27	3.66
18:0	18:2	18:1	1.70	0.53	0.43	1.69	0.77	0.77
18:0	18:1	18:2	0.77	0.52	0.44	0.69	0.77	0.77
18:1	18:1	18:1	16.3	13.1	8.87	13.03	13.19	8.03
18:1	16:0	18:2	–	3.5	3.9	0.43	3.27	3.66
18:1	18:0	18:2	–	0.52	0.59	0.19	0.77	0.77
18:1	18:2	20:0				0.95	0.40	0.44
18:1	18:2	22:0				1.63	0.73	1.07
18:1	18:2	24:0				0.45	0.31	2.05
18:2	18:1	20:0				0.55	0.40	0.44
18:2	18:1	22:0				0.98	0.73	1.07
18:2	18:1	24:0				0.44	0.31	2.05
16:0	18:2	18:2	2.82	2.11	3.3	2.58	1.79	3.39
18:0	18:2	18:2	0.56	0.31	0.40	0.63	0.42	0.71
18:1	18:2	18:1	11.8	7.9	7.42	11.85	7.22	0.10
18:1	18:1	18:2	9.1	15.9	15.9	9.89	14.46	14.84
18:2	16:0	18:2	–	1.06	1.74	0.06	0.89	1.69
18:2	18:0	18:2	–	0.16	0.26	0.03	0.21	0.35
18:1	18:2	20:1				0.81	0.37	0.12
18:1	20:1	18:2				–	0.37	0.12
18:1	18:2	18:2	6.6	9.6	13.8	8.99	7.92	13.72
18:2	18:1	18:2	1.9	4.8	6.82	1.50	3.96	6.86
18:2	18:2	20:1				0.39	0.20	0.11
18:2	20:1	18:2					0.10	0.06
18:2	18:2	18:2	1.4	2.9	6.10	1.36	2.17	6.35

[a]Abbreviations: PNO = native peanut oil; PNOR = interesterified peanut oil, PGF = mixture of cottonseed (10%), olive (55%), and safflower (35%) oils calculated to resemble peanut oil minus the long chain fatty acids (20:0; 22:0; 24:0); PGFR = PGF interesterified with arachidic and behenic glycerides synthesized to resemble PNO; CO = corn oil.

[b]Reconstituted estimates were obtained from the gas liquid chromatographic resolution of diacylglycerols generated by Grignard reaction.

[c]Calculated estimates were obtained from calculation of 1-random, 2-random, 3-random distribution for PNO and 1,2,3-random distribution for PNOR and PGFR.

Reconstitution of Molecular Species of Triacylglycerols

Table VI gives the molecular species composition of the triacylglycerol mixtures of the native, interesterified, and synthetic peanut oils. The estimates for the various species are represented as a sum of the racemates, although the native oil frequently contained only one enantiomer of a particular racemic pair. The reconstituted values for the native (PNO) and rearranged (PNOR) peanut oil were obtained by taking the experimental composition of the X-1,3-diacylglycerols for each of the oils and fitting the fatty acid composition of the 2-position by trial and error to approximate the pairing of the fatty acids in the *sn*-1,2(2,3)-diacylglycerols and the molecular weight distribution of the triacylglycerols in the appropriate silver nitrate fractions, all of which were also determined experimentally. Since the SILAR 5CP columns did not give valid estimates of the high molecular weight X-1,3-diacylglycerol species, the quantities of the corresponding long chain triacylgycerols could not be directly reconstituted. An estimate of them, however, could be obtained by calculation.

The calculated values for the molecular species of the triacylglycerols in the native peanut oil were obtained by *sn*-1-random, 2-random, 3-random calculation, while the calculated values for the rearranged and synthetic peanut oils were obtained by 1,2,3-random calculation.

There is close agreement between the reconstituted and the calculated composition of the molecular species of the rearranged and synthetic peanut oils, with any minor differences attributable to experimental error. This establishes further the validity of the analytical approach and adds credibility to the

differences noted between the compositions of the molecular species of the native and rearranged oils derived by both reconstitution and calculation. There is evidence that the differences between the reconstituted and calculated composition of the molecular species of the native oil are somewhat greater than those between the calculated and reconstituted compositions of the molecular species of the rearranged and synthetic oils. These differences could have arisen from a nonrandom molecular association of the fatty acids in the native oil. The significance of these differences, however, was not further investigated.

As a result of these analyses, it is seen that the native and interesterified peanut oils differ largely in the higher content in the native oil of *sn*-glycerol-1-palmitate, 2-linoleate, 3-oleate; *sn*-glycerol 1-oleate, 2-linoleate, 3-oleate; and the *sn*-glycerol-1-plamitate (oleate), 2-linoleate, 3-arachidate (behenate or lignocerate). About one-half of the total linoleate was present in the 2-position of the native triacylglycerols, while the randomized oil contained only about 30% of the total linoleate in the 2-position of the triacylglycerols. Furthermore, the triacylglycerols containing the C_{20}, C_{22} and C_{24}, and $C_{20:1}$ fatty acids were made up almost exclusively of the enantiomers with the long chain acids in the *sn*-3-position, while the rearranged oil contained these acids equally in all three positions. Since the rearranged oil also contained triacylglycerols made up exclusively of long chain fatty acids, the total proportion of triacylglycerols containing at least one long chain fatty acid per triacylglycerol molecule was significantly lower in this oil than in the native oil. This difference is further exaggerated if the enantiomeric nature of the acylglycerols is also considered and emerges as one of the major structural alterations brought about by randomization of the native peanut oil (compare calculated PNO and PNOR values in Table VI).

DISCUSSION

Previous dietary studies with native and interesterified fats have demonstrated great differences in the relative atherogenicity of native and rearranged peanut oil, which have been attributed to differences in their triacylglycerol structure (2). The present detailed investigation of the triacylglycerol structure of these oils has allowed the identification of the major molecular species, which although not unique to the native oil, are nevertheless present there in much higher proportions than in the interesterified preparation of the same fatty acid composition. These potentially atherogenic triacylglycerols are characterized by the presence of linoleic acid in position 2 and palmitic and oleic acids in positions 1 and 3. In addition, there were triacylglycerols containing linoleic and oleic acids in positions 1 and 2 with the long chain saturated acids in position 3. These triacylglycerols may be prepared synthetically and their relative atherogenicity determined in appropriate dietary experiments. These triacylglycerols may prove much more atherogenic than other triacylglycerols of comparable fatty acid composition, but of a much more random distribution of the acids. Likewise, a randomization of the synthetic triacylglycerols might result in a relative loss of their atherogenicity.

Should the synthetic triacylglycerols containing the linoleic acid in the 2-position prove to be more atherogenic than those of a more random distribution of linoleic acid but the same overall composition of the fatty acids, it would be interesting to speculate about the possibility that the relative atherogenicity of the native peanut oil is due to the relative inaccessibility of the linoleic acid to enzymic hydrolysis. Since the lipases involved in the hydrolysis of the dietary and chylomicron triacylglycerols are specific for the 1- and 3-positions of the triacylglycerol molecules, it is possible that the linoleic acid in the 2-position remains largely in the ester form throughout the processes of digestion, absorption, and clearance from plasma. This relative metabolic unavailability of the linoleic acid in the 2-position would render it metabolically much more saturated than other oils containing the same amount of linoleic acid in the external positions of the triacylglycerol molecule. This possibility is supported by the observation that both corn oil and the PGF fat contain both oleic and linoleic acids to about the same extent in the three positions of the triacylglycerol molecule, or at least to a proportionally much greater extent in the outer positions than the native peanut oil. Therefore, an interesterification cannot make these oils any less atherogenic by releasing linoleic acid from the internal to the external positions of the triacylglycerol molecule. The relative distribution of the linoleic acid between the inner and outer positions of the triacylglycerol molecules would appear to be consistent with the relative degree of atherogenicity of those oils tested. The generality of this observation could be extended to other oils by further dietary testing. In fact, a specific placement of the polyunsaturated fatty acids in the outer positions of the triacylglycerol molecule would allow theoretically the realization of minimum

atherogenicity per molecule of linoleic acid present.

The above discussion leaves unexplained, of course, the mechanism of action of the unsaturated oils in lowering plasma cholesterol and decreasing the atherogenic potential of a dietary fat. Should this aspect involve a protein-lipid interaction at the level of lipoproteins, it might be of interest to examine also the importance of the pairing of linoleic acid in position 2 with palmitic and oleic acid in positions 1 and 3 of the triacylglycerol molecule, as well as the diacylglycerol intermediates of the anabolic and the catabolic processes of triacylglycerol metabolism and transport. In any event, the identification of characteristic triacylglycerols of native peanut oil should provide useful clues to the nature of the biochemical basis of potential atherogenicity of natural fats and to practical methods for its elimination or suppression.

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Skin Surface Lipids of the Dog

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ABSTRACT

The skin surface lipid of the dog has been reported to contain a high proportion of diol diesters having a lower mobility on thin layer chromatography than diesters from other species in spite of containing similar fatty acid and diol components. In the present study, dog skin surface lipid was separated by preparative thin layer chromatography into sterol esters (42%), wax diesters (32%), free sterols (9%), polar lipids (7%), and unidentified components (10%). The diesters contained 1,2-diols, each esterified with one long chain fatty acid and one isovaleric acid moiety. The diols were principally branched chain C_{21} and C_{22} compounds while the long chain fatty acids esterified with them were mainly C_{20} and C_{21} branched compounds. The fatty acids from the sterol esters were mostly saturated, branched chain C_{19} to C_{23}, together with 7% of straight chain monoenoic acids, principally C_{21} and C_{22}. There were only trace amounts of free sterols other than cholesterol, while the esterified sterols contained 96% cholesterol and 4% lathosterol.

INTRODUCTION

Previous studies (1-6) have shown that the skin surface lipids of many mammals contain compounds which migrate on thin layer chromatograms in the region expected for aliphatic diesters. In the cow (5), the rabbit (5,6), and the domestic cat (5,6), these compounds contain 2-hydroxy acids esterified with a long chain fatty acid and a long chain alcohol (Type I diesters). In the rat (5), mouse (5,6), and guinea pig (3,5,6), the diesters have somewhat lower thin layer chromatography (TLC) mobility and are composed of long chain 1,2 -diols esterified with 2 equivalents of long chain fatty acids (Type II diesters). In dog skin lipids, a major component has a TLC mobility intermediate between Type II diesters and glyceryl ether diesters, although the hydrolysis products were reported to contain only long chain diols and long chain fatty acids (5,6). Thus, the differing TLC mobility of the Type II diesters from dog skin remained unexplained. We have now reinvestigated the diesters of dog skin surface lipids and find that each diol moiety is esterified with one long chain and one short chain fatty acid, 3-methylbutyric acid. We have also examined the composition of the free sterols and the sterol esters, which are the other major constituents of the dog skin surface lipids.

METHODS AND RESULTS

Materials

Previous investigations of sheep (7) and guinea pig (3) surface lipids provided long chain 1,2-diols, glyceryl ethers and fatty acid methyl esters of known composition. Pure 1-0-tetradecyl- and 1-0-octadecylglyceryl ethers, methyl palmitate, methyl stearate, and methyl oleate were purchased from Applied Science Laboratories, State College, PA. 3-Methylbutyric acid was purchased from Sigma Chemical Co., St. Louis, MO. 2-Methylbutyric acid was prepared by hydrogenation (H_2/Pt) of 2-methyl-2-butenoic acid (Sigma).

Collection of Surface Lipids

Hair (10 g) was clipped from a large dog (a female Collie) with scissors and extracted by immersion in chloroform:methanol (2:1) overnight. The hair and other debris were collected on a sintered glass filter, and the solvent was removed from the filtrate on a rotary evaporator at 40 C. The lipid residue (160 mg) was redissolved in hexane for analysis. For comparison of lipid class composition, smaller amounts of lipid were similarly extracted from hair combed from an Australian terrier, a Maltese, a Springer spaniel, and an Irish setter.

Separation of Lipid Classes

Preliminary TLC analysis using several solvent systems indicated the presence of several neutral lipid constituents (Fig. 1), which were resolved by development with benzene and then quantitated by charring and densitometry (8), as shown in Figure 2. The hair lipids from all five breeds had virtually identical lipid class compositions. Quantities of the constituents sufficient for identification and detailed analysis were obtained from the lipids of

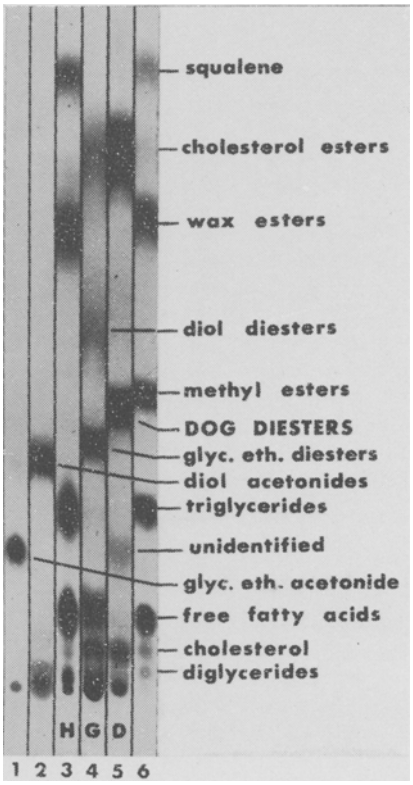

FIG. 1. Thin layer chromatograms of some skin surface lipids and derivatives. Lane 1: Isopropylidene derivative (acetonide) of 1-0-octadecylglyceryl ether. Lane 2: Isopropylidene derivatives of long chain 1,2-diols from dog skin wax diesters. Lane 3: Human (H) skin surface lipids. Lane 4: Guinea pig (G) skin surface lipids. Lane 5: Dog (D) skin surface lipids. Lane 6: Reference mixture. The chromatograms, on Silica Gel G, were developed successively with hexane (to 19 cm), benzene (to 19 cm) and hexane:ether: acetic acid (70:30:1, to 9 cm), and then visualized by charring.

the Collie by preparative TLC developed with benzene.

Wax Diesters (Peak 4, Fig. 2)

Diols: The structures of the dihydric alcohols, which were not established in previous studies, were investigated as follows. A sample of the wax diester fraction was saponified by heating in 0.5N NaOH in 95% methanol: benzene (2:1) at 50 C for 1 hr. The recovered unsaponifiable material was treated with acetone/H_2SO_4 to produce the diol isopropylidene derivatives. These were subjected to analytical TLC on Silica Gel G with hexane: ether:acetic acid (70:30:1) and had the same mobility as the corresponding derivatives of 1,2-diols from wool wax and guinea pig surface lipids, whereas, as expected (9) the isopropylidene derivatives of 1-0-alkylglyceryl ethers had somewhat lower mobility. The same solvent system was used for analytical TLC of the isopropylidene derivatives on silica gel /$AgNO_3$, which showed an absence of unsaturation. The chain length distribution and quantitation (Table I) were obtained by gas liquid chromatography (GLC) on SE-30 at 180 and 240 C and with temperature programming at 150-300 C at 3 C/min using the isopropylidene derivatives of 1,2-diols from the sheep and the guinea pig as reference compounds.

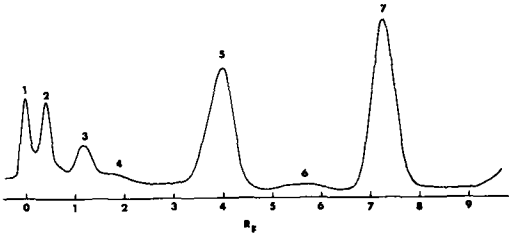

FIG. 2. Photodensitometer scan of a thin layer chromatogram of dog skin surface lipids on Silica Gel G developed with benzene and visualized by charring. Peak 1 = origin; 2 = cholesterol (9%); 3,4 = unidentified (7%); 5 = diol diesters (32%); 6 = unidentified (3%); 7 = sterol esters (42%).

A sample of the isopropylidene derivatives was boiled briefly in ethanol/HCl and 10 mg of the recovered diols in 1 ml *tert*-butanol was added to 2 ml water containing 40 mg $NaIO_4$ and shaken overnight. TLC indicated quantitative oxidation to aldehydes. These were oxidized in 1 ml acetone with several drops of 8N CrO_3 in H_2SO_4 (10), and the resulting fatty acids were converted to methyl esters with BCl_3/methanol. GLC analysis showed a chain length distribution pattern similar to that of the original diols but with corresponding peaks containing one less carbon atom, establishing the 1,2-location of the original hydroxyl groups.

Fatty acids: After the saponification of wax diesters described above, the recovered fatty acids were esterified with BCl_3/methanol. Analytical TLC on silica gel/$AgNO_3$ and GLC revealed only saturated long chain acids (Table I), providing no explanation for the low TLC mobility of wax diesters.

To investigate the possibility that a component of the wax diesters had escaped detection after hydrolysis, a sample of the wax diesters was transesterified with BCl_3 in methanol/benzene. The solvents were then evaporated and the residue was treated with pyridine/acetic anhydride. The resulting mixture of fatty acid methyl esters and diol diacetates was collected in hexane. A similar procedure was applied to synthetic mixtures of 1,2-diols and stearic acid in 1:1 and 1:2 mole

TABLE I

Composition (wt %) of the Aliphatic Components of the Wax Diesters and Sterol Esters of Dog Skin Surface Lipids

	Wax diesters								Sterol ester fatty acids			
	1,2-Diols			Long chain fatty acids					Saturated (93%)			Monoenes (7%) (straight)
	Straight	Branched		Straight	Branched				Straight	Branched		
Number of C atoms	%	ECL[a]	%	%	ECL	%	ECL	%	%	ECL	%	%
14				0.8	13.63	0.6			0.1	13.65	0.2	
15	tr[b]	14.56	tr	0.5	14.65	3.2			tr	14.67	6.8	
16	tr	15.55	tr	12.6	15.63	0.9	15.28	0.6	0.6	15.68	0.9	
17	1.0	16.57	tr	0.1	16.67	1.5			0.1	16.67	1.6	
18	1.0	17.60	6.5	1.9	17.64	0.6			2.2	17.67	2.6	
19	0.8	18.63	12.0	0.7	18.64	8.3			0.5	18.67	13.4	
20	9.0	19.63	6.2	1.9	19.70	25.5	19.30	0.3	3.7	19.67	15.2	4.0
21	0.8	20.69	22.0	0.6	20.72	28.0	20.28	0.9	0.4	20.67	21.7	31.3
22	0.9	21.60	22.5	0.1	21.63	4.4	21.29	1.1	1.8	21.64	9.4	42.5
23	tr	22.67	14.0	0.6	22.62	1.0	22.31	0.2	0.1	22.60	7.5	8.8
24	tr	23.67	2.4	0.2	23.61	0.5	23.30	tr	0.8	23.58	3.0	4.9
25	0.9	24.65	tr	0.7	24.61	0.6	23.29	tr	tr	24.57	2.9	6.3
26	tr	25.68	tr	tr	25.62	0.6	24.28	tr	0.2	25.63	1.3	2.1
27					26.63	0.5			tr	26.68	1.0	
28									tr	27.65	0.4	
29									tr	28.60	0.5	
30										29.64	0.7	
31										30.60	tr	
32										31.66	0.4	
Total	14.4		85.6	20.7		76.2		3.1	10.5		89.5	99.9

[a]Equivalent chain length.

[b]tr = <0.1%.

ratios, to serve as reference standards. Each of the three mixtures was then analyzed by quantitative TLC (8), which revealed that the products from the dog wax diesters contained one equivalent of long chain fatty acids per equivalent of diols rather than the two equivalents of acids required for diester formation.

That the missing acyl moiety was not acetate was demonstrated by synthesis of diesters in which each diol molecule was esterified with one palmitoyl and one acetyl group, giving diesters of lower TLC mobility than the natural compounds. The presence of other low molecular weight esterifying acids was investigated by carrying out hydrolysis, esterification, and GLC analysis of the diesters under conditions where volatile acids would not be lost (11-13). Thus, after saponification with 0.5N NaOH in 30% *tert*-butanol, the mixture was acidified and extracted twice with hexane, which collects fatty acids as short as butyric acid quantitatively (13). The combined hexane extracts were then shaken with 1% tetramethylammonium hydroxide, and an aliquot of this solution was dried at 120 C in a capillary probe and injected into the gas chromatograph flash heater at 300 C. The tetramethylammonium salts were thereby pyrolized quantitatively to methyl esters (11,12) which were analyzed by raising the temperature of the GLC column from 25 C to 200 C at 10 C/min. This showed the presence of a major component emerging at the same position as authentic methyl isovalerate. Quantitative GLC analysis of the mixture of short and long chain fatty acids indicated that the isovaleric acid contributed 68 mole % of the mixture, suggesting that some diisovaleryl esters may have been present initially. However, such esters were prepared synthetically and had a much lower TLC mobility than the native diesters, such that they would not have been included in the diester fraction from dog skin lipids isolated by preparative TLC. Thus, for reasons that are unclear, the GLC quantitation of isovaleric acid must have been inaccurate.

To prepare a quantity of the volatile fatty acid for further identification, a sample of the wax diesters was saponified and then subjected to steam distillation to remove any volatile, nonacidic materials. The residue was then acidified with H_2SO_4 and steam distillation continued. The distillate was made alkaline with NaOH and evaporated to dryness, and the residue was treated with BCl_3/methanol in a sealed tube. After 18 hr, the solution was diluted with water, and the methyl ester was extracted into ether. Aliquots of the ethereal solution were then fractionated by gas chromatography at 70 C on a 6 ft x 2 mm column packed with 20% DEGS. The emerging methyl ester was introduced directly into a mass spectrometer (Perkin-Elmer/Hitachi Model 6E). Spectra were also obtained for the methyl esters of 2-methyl- and 3-methylbutyric acids. The spectra of the methyl esters of the natural compound and 3-methylbutyric acid were virtually identical and distinctly different from that of 2-methylbutyric acid methyl ester (Table II).

TABLE II

Principal Ions Obtained in the Mass Spectra of the Methyl Esters of 2-Methyl- and 3-Methylbutyric Acids and the Volatile Fatty Acid Obtained from the Wax Diesters of Dog Skin Surface Lipids

	Relative abundance (% of base peak)		
m/e	3-Methyl	2-Methyl	Dog
116	5	5	4
101	18	43	17
88	14	100	6
85	42	70	35
74	100	5	100
59	40	45	61
57	46	100	34

Free Sterols and Sterol Esters

Sterols: GLC and TLC analysis of the free sterols (Peak 2, Fig. 2) and the coloration produced during charring of the TLC chromatograms, indicated that the free sterols consisted almost exclusively of cholesterol (99%). The sterols obtained on saponification of the sterol esters (Peak 7, Fig. 2) consisted predominantly (96%) of cholesterol. A minor sterol component (4%) had chromatographic properties identical with those of lathosterol. In both the free and esterified sterols, there were trace amounts of what appeared to be the C_{28-30} intermediates of sterol biosynthesis.

Fatty acids: The methyl esters of the fatty acids from the sterol esters were separated into saturated (93%) and monounsaturated (7%) fractions by preparative TLC on silica gel/$AgNO_3$, and both fractions were analyzed by GLC. The saturated fatty acids were predominantly branched chain, whereas the unsaturated acids were entirely straight chain compounds (Table I).

Unidentified Constituents

Peak 1, Fig. 2: This fraction of the surface lipids, which would have contained any polar lipids and free fatty acids, was not examined.

Peak 3 and 4, Fig. 2: These materials appeared to consist of esters bearing a free hydroxyl group. Hydrolysis yielded a complex mixture of fatty acids, hydroxyacids, fatty

alcohols, long chain diols, cholesterol and hydroxysterol, perhaps 7-hydroxycholesterol. This mixture was not analyzed in detail, but no isovaleric acid was detected.

Peak 6, Fig. 2: This component was not examined, but from its TLC mobility, it could have contained diesters in which the long chain diols were esterified with two long chain fatty acids.

DISCUSSION

The quantitative TLC analysis of the mixture of diol diacetates and fatty acid methyl esters obtained from the dog surface lipid diesters demonstrated that each diol was originally esterified with one long chain fatty acid. Synthetic diesters containing two long chain acids were well separated on TLC from the major diester component and coincided with a minor component (Peak 6, Fig. 2). Likewise, synthetic diisovaleryl esters of the diols were well separated on TLC, at a lower R_f than the natural diesters. Thus, in the major diester component, each 1,2-diol must be esterified with one long chain and one short chain fatty acid.

The previous reports noted a curious similarity in the average molecular weights of all of the surface lipid diester waxes and triglycerides, as calculated from their components, falling in the relatively narrow range of 828 to 892 (1). The present finding that the diester waxes from the dog contain one equivalent of a 5-carbon acid considerably reduces the average molecular weight of these compounds (to about 700) and eliminates the apparent uniformity in molecular weight between species.

The composition of the sterol esters of dog skin surface lipids does not appear to have been reported previously. As in most mammalian surface lipids (2), the fatty acids in these esters are predominantly saturated, branched chain compounds of both odd and even carbon number.

The present investigation further extends the observation that each mammalian species has characteristically different composition of skin surface lipids. The most striking difference in dog skin lipids is the presence of a high proportion of volatile acid in the wax diesters. In addition, there is a notable absence of wax monoesters and of intermediates of cholesterol biosynthesis, both of which have come to be regarded as characteristic components of mammalian surface lipids.

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Heart Mitochondrial Metabolism after Feeding Herring Oil to Rats and Monkeys

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ABSTRACT

Heart mitochondrial oxidation of palmityl CoA and pyruvic acid was studied in rats and in the monkey *Macaca fascicularis* to determine the effects of feeding partially hydrogenated herring oil. Herring oil glycerides contain cetoleic acid (*cis*-11-docosenoic) which could have a similar effect to erucic acid (*cis*-13-docosenoic) in causing a rat cardiomyopathy. The initial rat heart mitochondrial response to dietary cetoleic acid (67% *cis*, 33% *trans*) was an in vitro decrease in palmityl CoA oxidation. Prolonged feeding of cetoleic acid mixture was associated with a significant metabolic adaptation, increasing pyruvate and palmityl CoA oxidation above control levels. In vitro addition of cetoleyl CoA (pure *cis* isomer) stimulated pyruvate dehydrogenase activity, a possible response to decreased β-oxidation. There was no significant adaptive change in pyruvate or palmityl CoA use in monkeys after prolonged feeding of partially hydrogenated herring oil. Cetoleyl CoA was a good substrate for monkey heart carnitine acyl transferase even in the presence of palmityl CoA. These observations suggest that C_{22} fatty acids may be matabolized more rapidly in monkey heart than in rat heart. Metabolic differences argue against using the rat as an experimental model for studying possible cardiotoxic effects of docosenoic acids in primates.

INTRODUCTION

Feeding either partly hydrogenated herring oil (HHO) or high erucic acid rapeseed oil causes similar heart lesions in laboratory rats (1). HHO contains cetoleic acid (*cis*-11-docosenoic), and erucic acid (*cis*-13-docosenoic) is found in some mustard and rapeseed oils. These docosenoic fatty acids are thought to be related to histiocyte infiltration and focal myocardial necrosis when fed to rats for several weeks (1-4). An initial heart lipidosis which is resolved after 2-3 weeks of docosenoate feeding suggests that some metabolic adaptation to these fatty acids occurs (5). The sequence of metabolic changes from the control condition to the acute lipidosis to the chronic heart lesions has not been studied in detail (5,6). Erucic acid is slowly metabolized to CO_2 by rat heart preparations (7,8), and it can also be stored as triglyceride or phospholipid (5,9). Therefore, several forms of the docosenoic acids should be considered in studying cardiotoxic effects. Free erucic acid and its carnitine and coenzyme A esters have been studied in in vitro heart preparations by several investigators (6-8, 10-11). Several different types of effects can be studied with in vitro addition of any docosenoate derivative:

1. Changes in heart metabolism in naive animals. This simulates the conditions which lead to the lipidosis.
2. Metabolic adaptations which restore normal substrate use in the presence of inhibitory docosenoate derivatives, or increase the oxidation or mobilization of docosenoate. These changes would help to resolve the lipidosis.
3. Impairment of normal metabolism which persists without in vitro addition of docosenoate. This would be a metabolic lesion analogous to the histological lesions.

The first and third types of effects have been studied intensively for erucic acid (7,8,10 and 5,12,13) while the second type has only received superficial study (6). We have investigated all three types of effects for cetoleyl coenzyme A in this study. The effects have been compared for rats and for the primate, *Macaca fascicularis* (cynomolgus monkey).

MATERIALS AND METHODS

Animals and Diets

The subjects for this study were weanling male Sprague Dawley rats, and 1-2 year-old wild-caught *M. fascicularis* of both sexes. Rats and monkeys were fed the same diets. The diets contained either a lard-corn oil mixture, 3:1 (w/w), or partly hydrogenated herring oil (HHO) as 25% of the weight or 55% of the

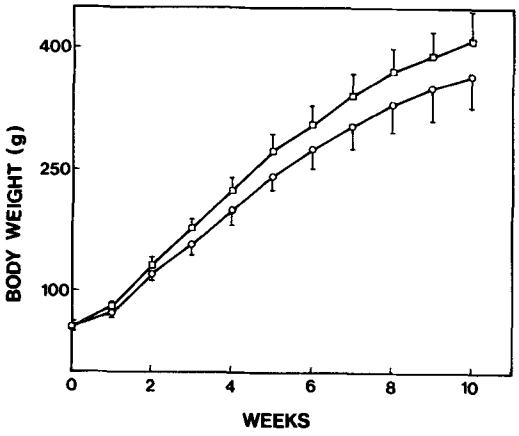

FIG. 1. Rat growth with diet containing partially hydrogenated herring oil (HHO). Control and HHO diets were fed ad libitum to individually caged rats and food consumption was recorded. Average weights with SD indicated for 20 rats fed the lard-corn oil control diet □———□. Average weights and SD for 10 rats fed the HHO diet ○———○.

calories. The cetoleic acid in this HHO was a mixture of 67% *cis* and 33% *trans* isomers. The combined docosenoate isomers accounted for 20% (w/w) of dietary fatty acids. The diet was formulated from cereal grains and supplemented with vitamins and minerals to meet the nutritional requirements of *M. fascicularis* (14).

Thirty rats were divided into three groups of ten. The control group was fed the lard-corn oil control diet, the chronic group was fed the diet containing HHO, and the acute group ate the control diet initially and the HHO diet for the final week of the experiment. The rat feeding trial lasted 10 weeks. Rats were sacrificed by decapitation and exsanguination. The hearts from two rats in each group were immediately placed in physiological saline at 2 C prior to pooling for mitochondrial preparation.

Monkeys were fed the experimental diets for 6 months. At the end of the feeding period, three monkeys from a control group and three fed the HHO diet were sacrificed by exsanguination under ketamine HCl anaesthesia. Samples of 500 mg to 1 g from the apex of the heart were chilled in physiological saline for mitochondrial isolation. Rat and monkey heart mitochondria were isolated accoarding to Pande and Blanchaer (15) using a bacterial protease. Heparin (300 units/ml) was included in the homogenizing buffer (13).

Enzyme Assays

Palmityl CoA oxidation was measured by the technique reported for the oxidation of palmitic acid by Swarttouw (8). The assay was modified by replacing palmitic acid and reduced CoA by 50 μM 1-[^{14}C]-palmityl CoA. The $^{14}CO_2$ released was absorbed in 250 μl of methyl benzethonium chloride and the activity measured in a liquid scintillation counter. Cetoleyl CoA (pure *cis* isomer) (50 μM) was included in some assays. This concentration was chosen for similarity to levels of fatty acid derivatives used by other investigators in in vitro oxidation systems (7-9). Cetoleyl CoA was preincubated for 5 min with the reaction system and mitochondria before addition of 1-[^{14}C]-palmityl CoA.

Carnitine acyl transferase was also assayed according to Swarttouw (8). This assay was changed by using 1-[^{14}C]-palmityl CoA to measure palmityl carnitine synthesis. Total acyl carnitines were quantitated by the [^{3}H]-carnitine used as the other substrate. The double label gave an estimate of competition between palmityl CoA and cetoleyl CoA as substrates for carnitine acyl transferase.

Pyruvate dehydrogenase activity was assayed as $^{14}CO_2$ production from 1-[^{14}C]-pyruvate in a 2.0 ml system containing 100 μmoles hydroxyethyl piperazine ethane sulfonate (HEPES) pH 7.2, 300 μmoles sucrose, 0.2 μmoles reduced CoA, 80 μmoles cysteine, 2 μmoles NAD, and a suspension of intact mitochondria containing 0.4 to 1.0 mg protein. The reaction was started by adding 4 μmoles (0.4 μCi) 1-[^{14}C]-pyruvate. The reaction system was incubated for 30 min at 37 C in a stoppered flask and stopped by adding 100 μl of 70% $HClO_4$. $^{14}CO_2$ absorption in 250 μl of methyl benzethonium chloride was completed by shaking the incubation flasks for 30 min

TABLE I

Effects of Partially Hydrogenated Herring Oil (HHO) Diets on Relative Heart Size and Lipid Content in Rats[a]

Diet treatment	Heart to body weight ratio	Total heart lipid % wet weight
Control	0.031	3.5
Acute HHO	0.033	7.9
Chronic HHO	0.032	3.8

[a]Values are averages from five pools with two rat hearts in each pool.

after addition of $HClO_4$. The mitochondrial suspension was omitted from the control incubation.

Oxidative Phosphorylation

Oxygen uptake was measured in a system containing 130 μmoles sucrose, 60 μmoles glucose, 100 μmoles KCl, 80 μmoles HEPES pH 7.4, 5 μmoles K_2HPO_4, 2 μmoles disodium EDTA, 2 μmoles $MgCl_2$, 10 μmoles of pyruvic acid, and 5 μmoles malic acid and mitochondrial suspension (0.5 to 1.0 mg of protein) in a volume of 2.0 ml. The system was held at 30C and O_2 uptake was measured by a Clark oxygen electrode. ADP/O ratios were measured from state 3 oxygen uptake after adding 0.50 μmole ADP. The respiratory control index was the ratio of state 3 to state 4 respiratory rates.

Other Methods

Purified *cis*-11-docosenoic (cetoleic) acid was purchased from Nu-Chek-Prep, Elysian, MN. Cetoleyl CoA was synthesized by Cheng and Pande's procedure for making erucyl CoA (11). Total heart lipids were measured by extracting an aliquot of the heart homogenates according to Bligh and Dyer (16). Protein was determined by Zak and Cohen's method (17). The statistical significance of diet treatments, day effects, and cetoleyl CoA inhibition were measured by analysis of variance, and error mean squares compared for significant differences using Duncan's Multiple Range Test (18).

RESULTS

Rats

There were no problems with acceptance or palatability of the diets. Food intake was the same with the HHO and the control diets. The control groups of rats gained weight faster than the HHO fed rats which may be due to a slightly greater food conversion efficiency, as digestibility and food efficiency may decrease with HHO diets due to reduced activity of pancreatic lipase with HHO (5). Weight gains for the two diet groups are shown in Figure 1. The rats in the acute group are included with controls since they ate the control diet until the final week of the experiment. There were no differences in appearance or behavior associated with consumption of the HHO diet.

The ratio of heart weight to body weight at the time of sacrifice is shown in Table I. There were no significant differences in these ratios of heart to body weight. The total lipid content of the hearts was elevated by acute HHO feeding, but returned to normal with 10 weeks of HHO diet (Table I).

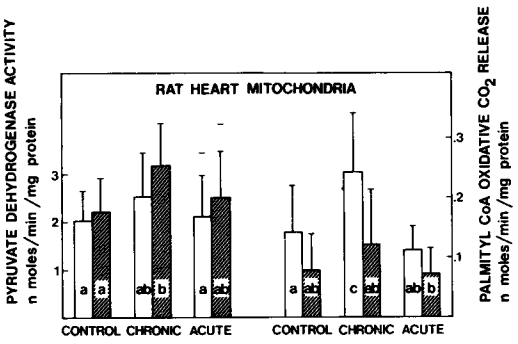

FIG. 2. Oxidative decarboxylation of pyruvate and palmityl CoA by rat heart mitochondria. $^{14}CO_2$ release was measured from 1-[^{14}C]-pyruvate (0.1 μCi/μmole) and from 1-[^{14}C]-palmityl CoA (0.8 μCi/μmole) as described in Methods. The open bars show activity with SD for the complete assay systems. The cross-hatched bars represent $^{14}CO_2$ release in the presence of 50 μM cetoleyl CoA. Cetoleyl CoA was added in an 8:1 molar ratio with fatty acid-free bovine serum albumin 5 min before the addition of ^{14}C substrates. Bars without a common letter are significantly different ($p < .05$).

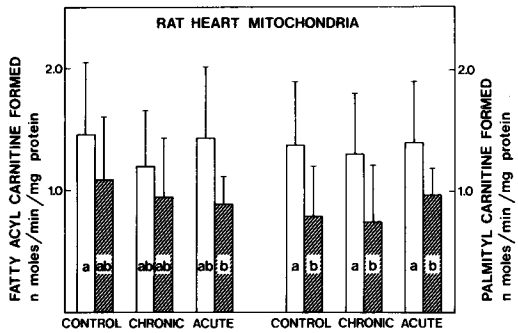

FIG. 3. Rat heart mitochondrial carnitine acyl transferase activity. The enzyme activity was determined by using both methyl-[^{3}H]-carnitine (0.1 μCi/μmole) and 1-[^{14}C]-palmityl CoA (0.2 μCi/μmole) as substrates. Fatty acyl carnitines synthesized were extracted in n-butanol (8) and ^{3}H and ^{14}C activity determined by liquid scintillation counting. Open bars represent activity and SD in the complete system. Cross-hatched bars show activity after a 3 min preincubation with cetoleyl CoA. (100 μM, 8:1 molar ratio with fatty acid-free bovine serum albumin). Bars without a common letter are significantly different ($p < .05$).

The ability of isolated mitochondria to use the main energy substrates for the heart is shown in Figure 2. The presence of HHO in the diet did not affect the release of $^{14}CO_2$ from 1[^{14}C]-pyruvate. The addition of cetoleyl CoA to the incubation system increased the utiliza-

TABLE II

Respiration and Coupling by Rat Heart Mitochondria after Consumption of Partially Hydrogenated Herring Oil (HHO)[a]

Diet treatment	State 3 respiration	State 4 respiration	Respiratory control index	ADP/O ratio
	μmoles O_2/min/mg protein			
Control	0.108	0.048	2.3	$2.69 \pm .37^A$
Acute HHO	0.099	0.041	2.6	$2.41 \pm .35^B$
Chronic HHO	0.108	0.045	2.5	$2.40 \pm .54^B$
P	0.14	0.14	0.32	0.016

[a]10 mM pyruvate and 5 mM malate were substrate for oxidation. State 3 respiration was measured after addition of 0.5 μmoles ADP. Values with different superscripts are significantly different at the indicated p value. n = 5, SD is indicated.

tion of pyruvate by heart mitochondria of chronic HHO-fed rats in comparision to the rates observed for the control group.

The oxidative decarboxylation of palmityl CoA was altered in mitochondria from rats fed HHO (Fig. 2). In comparison to oxidation values from the control diet, acute HHO feeding caused a slight decrease, and chronic HHO feeding significantly increased the release of $^{14}CO_2$ from 1-[^{14}C]-palmityl CoA. Addition of cetoleyl CoA to these mitochondria reduced the oxidative decarboxylation of palmityl CoA by ca. 45% for each diet treatment. Cetoleyl CoA may resemble erucyl carnitine in inhibiting β-oxidation of palmityl derivatives (7,9), or it may act as a competing substrate for β-oxidation. This apparent inhibition was only statistically significant for chronic HHO-feeding. The effect of the adaptation in the oxidative decarboxylation of palmityl CoA with chronic HHO feeding can be shown by comparing the rates of oxidation in the control without cetoleyl CoA to the chronic HHO plus cetoleyl CoA. The adaptation *returned palmitate use to normal levels in the presence of cetoleyl CoA.*

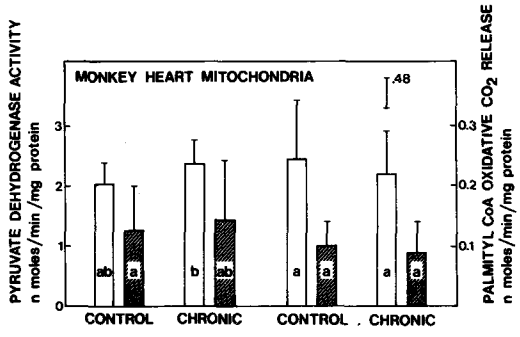

FIG. 4. Oxidative decarboxylation of pyruvate and palmityl CoA by *Macaca fascicularis* heart mitochondria. Conditions as in Figure 2. The open bars show activity with SD for complete assay systems. The closed bars show the effect of a 5 min preincubation with cetoleyl CoA. Bars without a common letter are significantly different ($p < .05$).

In contrast to observations made with erucyl carnitine (7), cetoleyl CoA inhibited the activity of carnitine acyl transferase in rat heart mitochondria (Fig. 3). Most of the carnitine ester formed was palmityl carnitine since the yield of fatty acyl carnitine was close in value to that of palmityl carnitine.

The respiratory properties of rat heart mitochondria are presented in Table II. The respiratory rates were not significantly affected by diet treatments. ADP/O ratios were significantly depressed in both chronic and acute HHO treatments, although a 10% decrease in ADP/O ratios may have little functional significance.

Monkeys

The data reported are from a group of monkeys sacrificed as part of a long term experiment on the effects of feeding partially hydrogenated herring oil. Information on clinical chemistry, myocardial physiology, histology, and gross pathology will be published elsewhere.

Pyruvate dehydrogenase activity in monkey heart mitochondria was not significantly affected by chronic HHO feeding (Fig. 4). Cetoleyl CoA addition to the assay system caused a substantial inhibition of pyruvate dehydrogenase in control and in chronic HHO-fed monkeys. Palmityl CoA oxidation was not increased by chronic HHO feeding in these monkeys (Fig. 4), and there was no evidence of an adaptive response to increase palmitate use.

Carnitine acyl transferase activity was not altered appreciably by feeding the HHO diet (Fig. 5). In marked contrast to the rat enzyme, cetoleyl CoA significantly increased total fatty acyl carnitine production in monkey heart mitochondria. The rate of acyl carnitine formation in monkey heart mitochondria was in considerable excess of the rate of $^{14}CO_2$ production from 1-[^{14}C]-

TABLE III

Respiration and Coupling by Monkey Heart Mitochondria after Consumption of Partially Hydrogenated Herring Oil (HHO)[a]

Diet treatment	State 3 respiration	State 4 respiration	Respiratory control index	ADP/O ratio
	μmoles O_2/min/mg protein			
Control	73 ± 7	40 ± 11	2.0 ± 0.6	2.42 ± .26
Chronic HHO	63 ± 18	27 ± 15	3.3 ± 2.9	2.56 ± .54
P	0.02	0.01	0.16	0.44

[a]Conditions were the same as for Table II. P values indicate the probability of these differences occurring due to chance alone. n = 3, SD is indicated.

palmitate. Both state 3 and state 4 oxidation rates for pyruvate and malate were significantly depressed by chronic HHO feeding (Table III), although pyruvate dehydrogenase activity was slightly elevated in the same mitochondria (Fig. 4). An increase in the respiratory control index with the HHO treatment was caused by the relatively low state 4 respiration rate. ADP/O ratios of monkey heart mitochondria were not affected by feeding HHO.

DISCUSSION

Feeding HHO to rats for 10 weeks caused a significant increase in the oxidative decarboxylation of palmityl CoA and a decrease in ADP/O ratios of heart mitochondria. Increased myocardial oxidation of palmityl CoA may represent a metabolic adaptation to docosenoate feeding (6), while depressed ADP/O ratios could arise from minor damage to the mitochondria which was not prevented by including heparin in the isolation medium (12,13).

In vitro addition of cetoleyl CoA to isolated rat heart mitochondria caused metabolic changes in mitochondria from naive rats and also revealed metabolic adaptations which could overcome effects of cetoleyl CoA after chronic HHO feeding. Pyruvate dehydrogenase activity was significantly increased in in vitro addition of cetoleyl CoA to mitochondria of rats fed the HHO diet. This appears to be an adaptive effect to maintain a constant O_2 uptake when fatty acid oxidation is reduced by cetoleyl CoA. Heijkenskjold and Ernster have reported a similar effect caused by erucyl carnitine (6). On the other hand, Christophersen and Christiansen found that pyruvate utilization in heart mitochondria from naive rats was inhibited by erucyl carnitine (10).

Chronic HHO feeding did not affect the 50-60% decrease in the rate of oxidative decarboxylation of palmityl CoA caused by adding equimolar cetoleyl CoA. The combined effects of the adaptive increase caused by chronic HHO feeding, and the decrease related to in vitro addition of cetoleyl CoA, resulted in rates of palmitate decarboxylation similar to those obtained from heart mitochondria of untreated rats. Cetoleyl CoA may act similarly to erucyl CoA as an inhibitor of carnitine acyl transferase activity on palmityl CoA (11). The most interesting effects of continuous HHO feeding to rats were the adaptations in pyruvate and palmitate metabolism which developed and persisted at the time when the recognized histological lesions are developing.

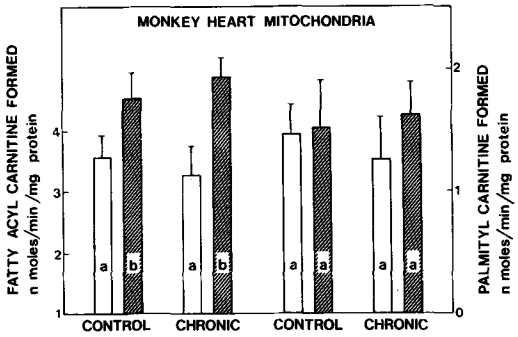

FIG. 5. *Macaca fasciularis* heart mitochondrial carnitine acyl transferase activity. Assay conditions as in Figure 3. Open bars represent enzyme activity and SD in the complete system. Cross-hatched bars show activity after a 3 min preincubation with cetoleyl CoA. Bars without a common letter are significantly different ($p < .05$).

The only effect of chronic HHO feeding to monkeys detected in this study was a slightly depressed oxygen uptake of heart mitochondria. In vitro addition of cetoleyl CoA in the pyruvate dehydrogenase assay depressed enzyme activity, opposite to the effect observed in the rat. Other in vitro effects of cetoleyl CoA were inhibition of palmityl CoA oxidation and a stimulation of acyl carnitine synthesis. One possible interpretation of these results could be a substantial oxidation of cetoleyl CoA by monkey heart mitochondria. This would explain the inhibition of pyruvate

dehydrogenase in the presence of a competing substrate, cetoleyl CoA. Since cetoleyl CoA promoted acyl carnitine synthesis, it may be more available for β-oxidation in the monkey than in the rat. Use of cetoleyl CoA of dietary origin as a substrate for β-oxidation in the monkey heart could account for the absence of adaptive changes in pyruvate and palmitate oxidation following chronic HHO feeding.

Rat and monkey hearts, therefore, have major differences in adaptive responses to chronic HHO feeding. Metabolic adaptation and heart lesions are associated in the rat although no causal relationship is obvious. Preliminary observations suggest that histological lesions are less severe in *M. fascicularis* than in laboratory rats after chronic HHO feeding (14), permitting the inference that lack of histological lesions is associated with the absence of metabolic adaptation to HHO in the monkeys. Additional comparative studies on cetoleyl CoA oxidation by heart mitochondria may clarify the suggested differences. Whatever the outcome of these studies, it is clear that metabolic differences make the rat a poor model for investigating myocardial effects of HHO diets in the primate, *M. fascicularis*, and potentially also in man.

ACKNOWLEDGMENTS

We wish to thank D.S. Hancock for excellent technical assistance and to acknowledge the financial support of the Fisheries and Marine Service of Environment Canada.

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A New System for Lipid Analysis by Liquid Chromatography-Mass Spectrometry[1]

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ABSTRACT

A simple system for interfacing liquid chromatography with mass spectrometry for the analysis of lipids is described. The system is based on the moving chain transport principle and employs an endless stainless steel belt of perforated construction that gives it superior surface properties and capacity to entrain solvent. The entire column eluent is collected on the belt which transports it into an evaporator where the solvent is removed. The solute, which remains as a residue on the belt, is transported into a reactor where it is converted to hydrocarbons by reaction with hydrogen at 400-450 C. The hydrocarbons, which are characteristic of the structures of the parent compounds, are swept into an outlet tube where ca. 15% are drawn into the ion source of a mass spectrometer operating in the chemical ionization mode using methane as the reagent gas. The spectrum is recorded on an oscillographic recorder for identification purposes. Detection and quantitative analysis is performed by single ion monitoring of the most intense ion using a conventional analog recorder. The system is demonstrated by application to a standard mixture of tripalmitin, cholesteryl palmitate, and cholesterol separated on a 3.2 x 250 mm silica column, and exhibited a sensitivity of ca. 1 nanogram per component injected on the column.

INTRODUCTION

Although the utility of liquid chromatography (LC) can be increased tremendously by interfacing it with mass spectrometry (MS), development of the technology has been slow. Most lipids, like many high molecular weight nautral products, do not lend themselves to volatilization without degradation because of their low vapor pressures and thereby are not readily amenable to mass spectrometry. Another problem encountered in interfacing LC with MS is the very low concentration of solute after the eluent is converted to the gaseous state as a carrier gas for intrdouction of the sample into the mass spectrometer. These problems and the techniques for dealing with them have been discussed recently by McFadden et al. (1). Although a number of LC-MS systems have been developed (1-6), none is applicable generally to the analysis of natural lipids.

Presented here is a system for interfacing liquid chromatography with mass spectrometry for the analysis of lipids based on the moving chain transport principle (7), as applied in this laboratory, to interface LC with a flame ionization detection system (8-10). In this system, the entire eluent from the LC column is collected by a moving belt of special construction; the solvent is removed by evaporation in a stream of nitrogen and the sample, as a residue on the belt, is transported into a reaction chamber where it is converted into hydrocarbons in a hydrogen-nitrogen atmosphere. The hydrocarbons are swept from the reaction chamber in the mixture of hydrogen and nitrogen or a mixture of hydrogen, nitrogen, and methane into a mass spectrometer operating in the chemical ionization mode. Most lipids yield hydrocarbons that provide structural identification as well as analysis in the low nanogram range.

EXPERIMENTAL PROCEDURES

Apparatus

A schematic of the interface system is shown in Figure 1. It represents a modification of that previously developed for interfacing LC with a flame ionization detection system (10). The applicator is the same as that used in the flame detector interface system (10). It consists of a Teflon tube, 0.8 mm ID, cut to fit the shape of the belt. The conveyor is a perforated stainless steel ribbon 125 cm long, 0.13 mm thick, and 1.60 mm wide that is welded by laser or electron beam techniques into an endless belt. It contains holes 0.18 mm in diameter at the large end and 0.28 mm on center in a 60° array with a solid border on each side ca. 0.20 mm wide as shown in Figure 2. The belt is fabricated by an etching process, and the surface is

[1]The work described herein was reported at the meeting of the American Oil Chemists' Society in New York, May 1977, Abstract #94.

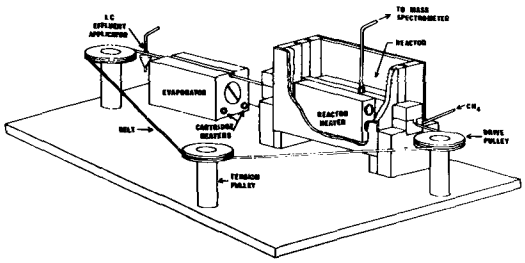


FIG. 1. Schematic of interface used to couple a liquid chromatograph to a chemical ionization quadrupole mass spectrometer.

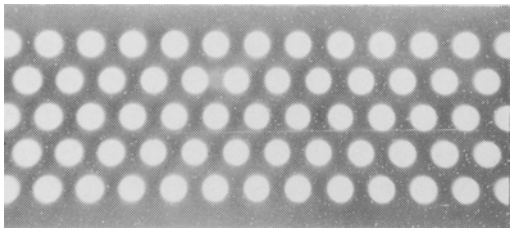


FIG. 2. Photomicrograph of endless conveyor belt.

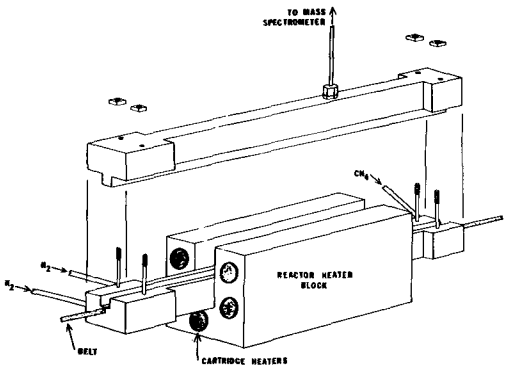


FIG. 3. Schematic of the reactor showing the sandwich type construction.

uniformly roughened by this process for better adhesion of the eluent. The evaporator is an aluminum block with a bored-out cavity for heating the nitrogen as it passes through a series of orifices into the channel through which the belt passes.

The reaction chamber is made of temperature resistant ceramic (Macor, Corning Glass Works, Corning, NY) and consists of upper and lower parts that fit together to give a gas tight sandwich. The lower section contains a channel 0.40 mm wide by 2.0 mm deep for the belt and is permanently aligned between the pulleys; the top section is removable to permit insertion of the belt into the channel. The central part of the unit, which is encased in an insulated compartment, is heated by cartridge heaters in a heating block that fits around it. Nitrogen is introduced into the channel of the forepart of the unit outside the reactor compartment to serve as a gas block and as a carrier gas to transport products of the reaction into the mass spectrometer. Hydrogen is introduced into the front end of the reactor as a reactant for the production of hydrocarbons; nitrogen or methane is used as a blocking gas to prevent escape of hydrogen or reaction products from the end of the channel. The products of the reaction in the carrier gas (hydrogen, nitrogen, and methane) are carried into an outlet tube in the center of the reactor where, by means of a splitter, 8 to 12 ml of the gas flow is directed into the source of the mass spectrometer operating in the chemical ionization mode.

The mass spectrometer used in this work was a Biospec Model 7401B. This instrument is a quadrupole type equipped with both EI and CI modes of operation and contains a solids probe injector. Normal operating parameters of this instrument when used in conjunction with the interface system are: an emission current of 0.24 milliamps, a pressure of 2.8 torr in the source, and a pressure of 2×10^{-5} torr in the analyzer. The mass spectra were recorded by means of an oscillographic recorder and/or an analog strip chart recorder in conjunction with a mass range selector. Methane was used as the reagent gas. Other gases, particularly isobutane, that do not give significant amounts of protonated oligo species can also be used.

The liquid chromatograph used in this study was a Spectra Physics Model 3500 B equipped with a variable wavelength detector, and a 3.2 x 250 mm 5 micron Spherisorb silica column (Spectra Physics Co., Palto Alto, CA).

The lipids used as standards were highly purified preparations (>99%) obtained from the Lipids Preparation Laboratory of The Hormel Institute; pentadecane (>98%) was obtained from Polyscience Corp., Inc., Niles, IL.

Operational Characteristics of the Interface System

Applicator: The Teflon tube that serves as an applicator is cut at the end so as to maintain a continuous wall of column eluent between it and the belt.

Transport device: The endless belt used as a vehicle to collect the column eluent has a capacity to retain solvent from the column at a flow rate of ca. 1 ml/min at a belt speed of ca. 2.0 cm/sec. In general, for the utmost in detector efficiency, the belt is operated at the speed that matches its full capacity to collect eluent from the column. For highest resolution in the

LC process, the solvent flow rate is optimized for highest column efficiency. Thus, for highest overall efficiency and resolution, the flow rate of the column determines the speed at which the belt is operated, provided this rate is within the capacity of the evaporator to remove solvent without loss of solute. For separation of the neutral lipids with the present system as reported herein, a flow rate of mobil phase of ca. 0.6 ml/min was used with a belt speed of 2.0 cm/min.

Belts of no. 316 stainless steel used in this study have a life of over 400 hr of continuous operation.

Evaporator: The purpose of the evaporator is to remove solvent from the belt without loss of solute. For the common lipid classes, the block is maintained at a temperature of 160-200 C and nitrogen is passed through it at 1000-1500 ml/min. Both temperature and flow rate of nitrogen are varied depending on the boiling point of the solvent and the relative volatility of the substances in the column eluent.

Reactor unit: The purpose of the reactor is to convert the solute, which is passed into it on the belt as a residue, to volatile hydrocarbons which are transported in the gaseous state in the carrier gas into the outlet tube. Ca. 10 ml/min of the carrier gas is drawn into the mass spectrometer through a capillary inlet tube, the length and diameter of which are standardized to this capacity. The transfer line to the mass spectrometer is maintained at ca. 250 C by wrapping it with electric heater tape. The conversion of the solute to hydrocarbons is effected by reaction with hydrogen at a temperature of ca. 430 C at which the reactor is maintained. The formation of hydrocarbons is believed to occur primarily by hydrogenolysis catalyzed by the chromium and nickel in the belt. Some reductive cracking also occurs, and this reaction is also probably catalyzed by the metal belt. The temperature of the unit is regulated so as to favor hydrogenolysis. The reason the belt is believed to act as a catalyst for the reaction is that chromium and nickel are well-known catalysts for these types of reactions. Under these conditions, hydrocarbons are produced that are characteristic of the structures of the compounds introduced into the reactor. Hence, the mass spectrometer is used not only as a detector, but also as a means of identification. For greatest sensitivity, the intensity of single ions are measured; for identification, the entire spectrum is generally recorded on an oscillographic recorder.

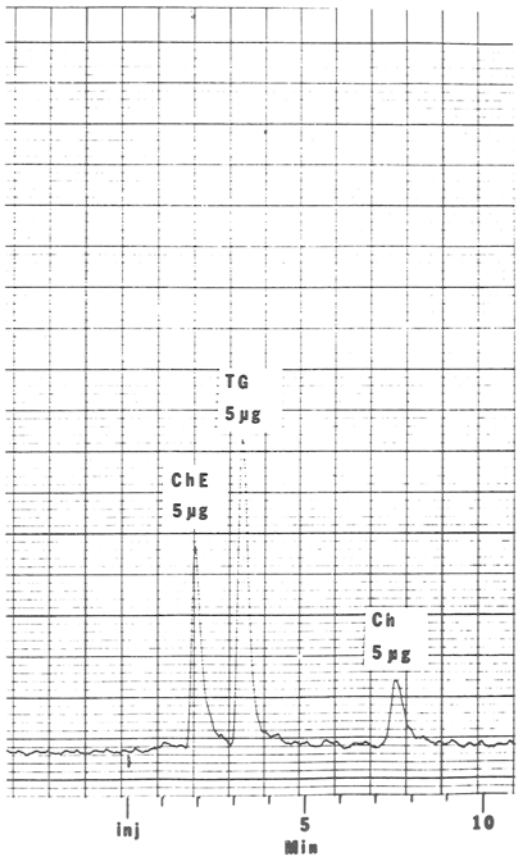


FIG. 4. Liquid chromatogram of neutral lipid standard mixture (ChE = cholesteryl palmitate, TG = tripalmitin, and Ch = cholesterol). Total ion monitor trace from m/e 95 to 400.

RESULTS

The performance of the interface system was demonstrated with a standard mixture of 5 µg each of tripalmitin, cholesteryl palmitate, and cholesterol separated on a 3.2 x 250 mm Spherisorb silica column (Fig. 4). In this analysis, the total ion current from 95 to 400 m/e was measured with a conventional analog recorder with a full scale deflection of 0.1 volts.

The mass spectrograms of the components of the mixture are shown in Figures 5, 6, and 7. The mass spectrogram in Figure 5 showed that the primary product obtained from tripalmitin was pentadecane, as evidenced by the strong intensity of the m/e 211 ion. Other fragments can be accounted for on the basis of further fragmentation of this hydrocarbon either in the interface system or in the mass spectrometer. In order to obtain an indication of the extent of secondary fragmentation in the interface system, the mass spectral analysis of pure

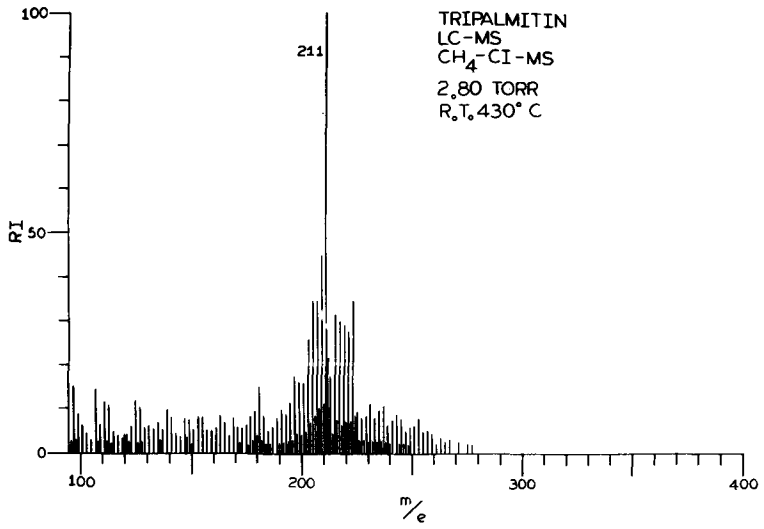


FIG. 5. Mass spectra of tripalmitin eluted from the LC column and analyzed by the mass spectrometer after reaction in the LC-MS interface system.

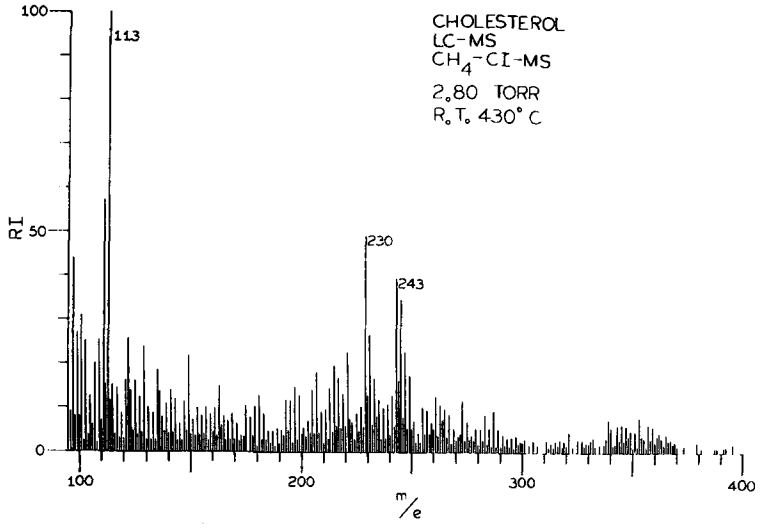


FIG. 6. Mass spectra of cholesterol eluted from the LC column and analyzed by the mass spectrometer after reaction in the LC-MS interface system.

pentadecane by a direct probe analysis at 60 C was compared with its analysis via the interface system (Fig. 8 and 9). These results showed that the minor fragments in the mass spectrogram of the tripalmitin came primarily from heptadecane. The greater degree of secondary fragmentation in the analyses of the sample introduced into the mass spectrometer from the interface system occurred primarily because of the high temperature (430 C) at which the reactor was operated. However, introduction of hydrogen into the mass spectrometer in this system also probably accounted for some of the increase in secondary fragmentation.

The products formed in the interface system from cholesterol and cholesteryl palmitate, Figures 6 and 7, respectively, have not been completely elucidated at present. However, they gave certain specific fragments characteristic of their structures. In the analyses of cholesterol, m/e ions at 230, 243, and 113 (Fig. 6) are characteristic. These ions also are present in the mass spectrogram of cholesteryl palmitate (Fig. 7), but the most intense ion for this

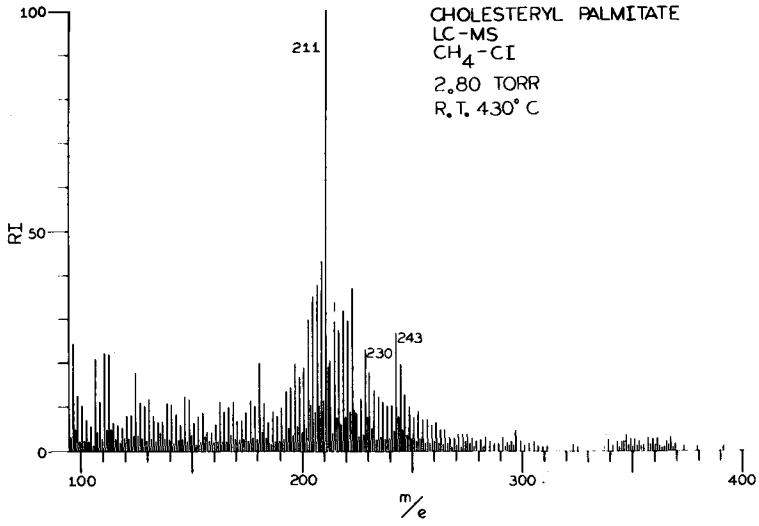


FIG. 7. Mass spectra of cholesteryl palmitate eluted from the LC column and analyzed by the mass spectrometer after reaction in the LC-MS interface system.

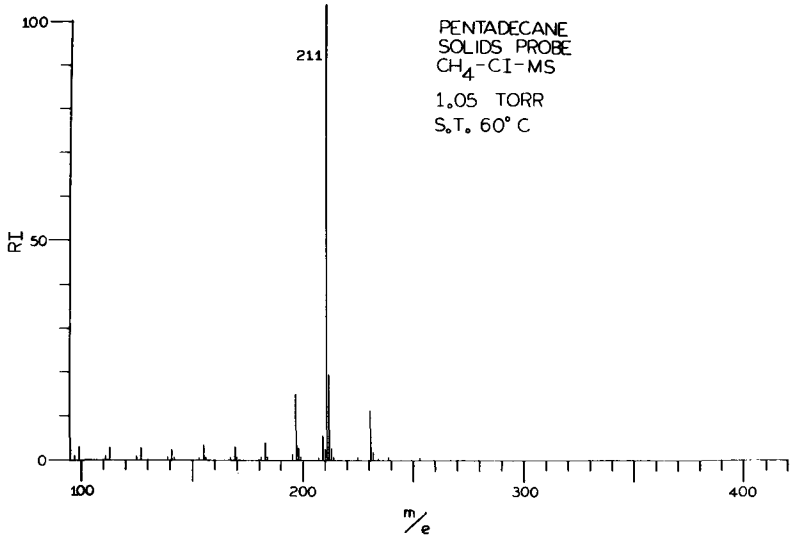


FIG. 8. Mass spectra of pentadecane analyzed via a solids probe at 60 C and 1.05 torr CH_4 source pressure.

compound is at 211 which is derived from the palmitate moiety by loss of CO_2.

By means of single ion monitoring of the m/e 211 ion, the sensitivity for the detection of tripalmitin and cholesteryl palmitate separated on the Spherisorb silica column was of the order of 1 nanogram, as shown in Figure 10. Studies on the possibility of using single ion monitoring as a method for the quantitative analysis of the lipid classes showed that the results with these compounds were highly reproducible and exhibited a broad linear range between response and mass, as shown in Figure 11.

DISCUSSION

The interface system described here is simple and durable and provides a sensitivity equivalent to that of an ultraviolet detector for strong UV absorbing compounds using the mass spectrometer as a universal detector. With further refinements in the system, particularly by the use of an appropriate separator, it should be possible to increase sensitivity inasmuch as only ca. 15% of the carrier gas is drawn into the mass spectrometer. Removal of hydrogen from the carrier gas should reduce secondary fragmentation in the mass spectro-

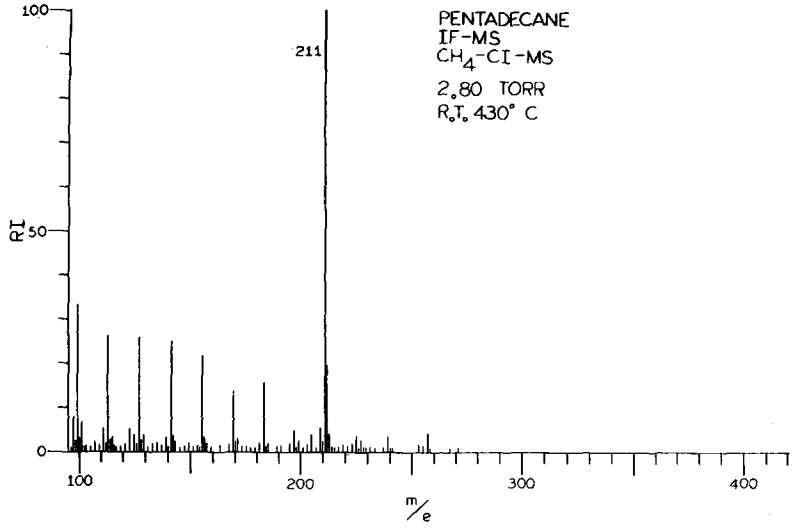


FIG. 9. Mass spectra of pentadecane analyzed by continuously applying a solution on the interface belt and operating the system under the same conditions as during the LC run.

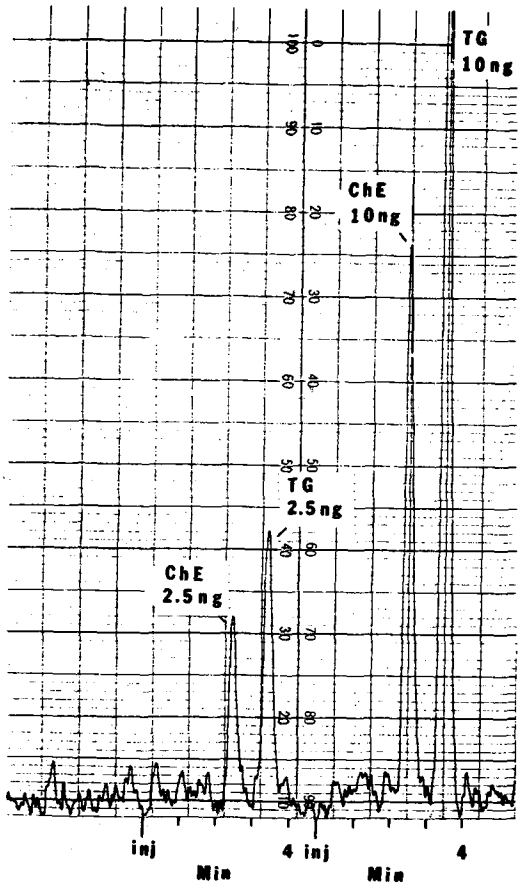


FIG. 10. Liquid chromatogram of neutral lipid standard mixture (ChE = cholesteryl palmitate and TG = tripalmitin). Single ion monitor trace at m/e 211.

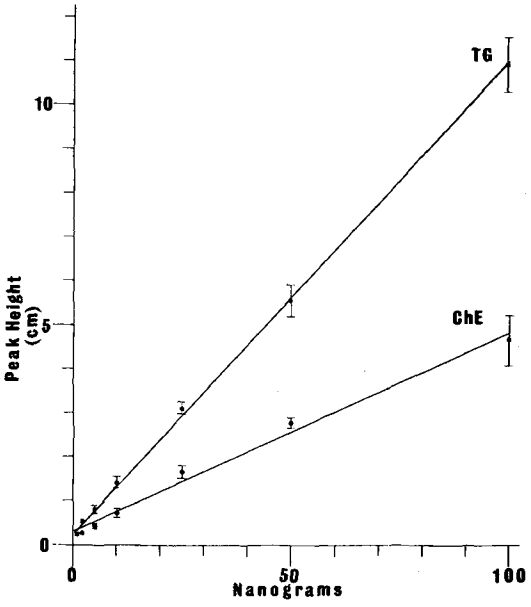
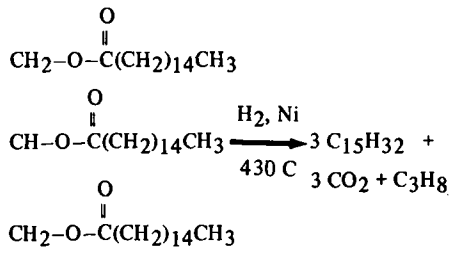


FIG. 11. Calibration curve for cholesteryl palmitate (ChE) and tripalmitin (TG) for samples injected on the LC column, reacted in the LC-MS interface system, and single ion monitored at m/e 211 in the mass spectrometer.

meter because it produces ions that are more reactive than those from methane.

With tripalmitin, the major product is pentadecane. This compound appears to be produced by hydrogenolysis according to the following reaction.



Comparison of the spectra of the pentadecane standard with the products from tripalmitin shows that, although fragmentation is greater, the pattern is essentially the same. No pyrolysis appears to occur under the conditions employed inasmuch as there is no evidence of the formation of free fatty acids which are a major product of the pyrolysis of triglycerides (11). Pentadecane is also produced from cholesteryl palmitate in the reactor, apparently by hydrogenolysis, as evidenced by the ion at an m/e of 211. With cholesterol, the major products appear to be formed by reductive cracking as evidenced by major ions at 113, 230, and 243 m/e. The mechanism of the formation of these ions has not been elucidated. However, they appear to be specific for cholesterol and might be used for quantitative analysis of this compound, particularly in an LC analysis via single ion monitoring.

The system described here does not provide a structural analysis, but the fact that the primary products can be associated with specific structures enables a structural identification to be made in many instances, particularly in conjunction with an LC separation. Moreover, with compounds that give highly specific products, a direct analysis may be made by mass spectrometry via the interface system without prior separation by LC.

At present, no studies have been made on the use of the system for quantitative analysis; however, the preliminary experiments reported in Figure 11 indicate that the response to mass is linear over a wide range.

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Lipid Metabolism of the Yellow Clam, *Mesodesma mactroides:* 3-Saturated Fatty Acids and Acetate Metabolism

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ABSTRACT

The fate of labeled palmitate, stearate, and acetate administered to the yellow clam, *Mesodesma mactroides,* was investigated. 1-^{14}C palmitic and 1-^{14}C stearic acids were oxidized to CO_2 to a limited extent. They were mainly incorporated in diacylglycerols and triacylglycerols and were converted to higher homologs. After administration, palmitic acid was converted to stearic and oleic acids, whereas administered stearic acid was converted to 18:1, 18:2, 20:1, and 20:2 acids. Labeled acetate was readily included by the clam in 12:0, 14:0, 14:1, 15:0, 16:1, 16:1, 16:2, 18:2, 18:1, 18:2, 20:1, 20:2, and 20:3 acids.

INTRODUCTION

In our previous work (1), the effect of food composition on the lipid and fatty acid composition of the yellow clam, *Mesodesma mactroides,* collected in different seasons on the beaches of Mar Azul, Argentina, was reported. It was also reported (2) that labeled linoleate, α-linolenate, and higher homologs of α-linolenic acid dissolved in the seawater were incorporated by the clam. Linoleic and α-linolenic acids were desaturated and to some extent elongated to higher homologs. The desaturation was enhanced by addition of casein hydrolysate to the medium. In spite of this transformation, the polyunsaturated fatty acid pattern of the clam preferentially reflected the fatty acid composition of the food.

To complete this study we have investigated in the present experiment the metabolic fate of labeled palmitic and stearic acid as well as the de novo synthesis of fatty acids from labeled acetate.

MATERIAL AND METHODS

Materials

Labeled acids were provided by Amersham-Searle (Amersham, England). The radiochemical purity was higher than 99%.

Organisms

M. mactroides were harvested in March on the sandy beaches of Mar Azul, Argentina, as described in a previous paper (1). They were kept in a net hung in aerated and filtered sterile seawater for 1 day before the experiment. By this procedure, planktonic contamination was practically eliminated.

Radioactive Fatty Acid Administration

As described previously (2), two types of media were used. Medium No. 1 was a synthetic sterile seawater solution. Medium No. 2 was prepared in the same way and supplemented with 1 g/liter Casenolin (casein hydrolysate, Glaxo, Buenos Aires, Argentina). The ammonium salts of the labeled acids were added in a proportion of 0.01 to 0.015 moles/100 ml of medium. 1-^{14}C sodium acetate was added in a proportion of 0.098 to 0.100 moles/ml.

Procedure

The procedure described by Moreno et al. (2) was used throughout the metabolic studies. Very little contamination with bacteria or other microorganisms is then possible. Two clams per bottle containing 800 ml of sterile medium were maintained at room temperature for specified periods of time and labeled CO_2 expired was collected and counted. The lipids of the clam were extracted as already described (2) and the radioactivity was measured. Labeling distribution in the fatty acids was studied by gas liquid radiochromatography. A Pye apparatus equipped with a proportional counter was used. Fatty acids were identified as described previously (1). The specific radioactivity of fatty acid methyl esters was determined in fractions separated on a F&M gas liquid chromatograph. The esters were collected in a scintillation solution of diphenyloxazole (PPO) and 5-phenyloxazolbenzene (POPOP) in toluene and counted in a Packard scintillation counter. The total radioactivity recovered was 70-72%. To calculate the relative specific activity of each fatty acid methyl ester, the radioactivity of each peak was corrected for 100% and was divided by the area of the peak

[1]Member of the Carrera del Investigador Científice of the Consoje Nacional de Investigaciones Científicas y Técnicas, Argentina.

TABLE I

Radioactivity of 1-^{14}C Palmitic and 1-^{14}C Stearic Acids Incorporated and Oxidized by *Mesodesma mactroides*

Fractions	1-^{14}C 16:0		1-^{14}C 18:0	
	Percent[b]	dpm/mg	Percent	dpm/mg
Total lipids	13.2 ± 1.9	1524 ± 106	15.0 ± 1.0	1770 ± 24
Fatty acids	11.5 ± 1.2	7612 ± 550	13.3 ± 0.8	8867 ± 621
Water soluble	0.1 ± 0.0	–	0.05 ± 0.01	–
Expired CO_2	0.2 ± 0.1	–	1.0 ± 0.3	–

[a]Labeled acids were administered dissolved in Medium No. 1 during 12 hr. Results are the mean of eight groups of two clams each ± SEM.

[b]The radioactivity of the medium before incubation was taken as 100%.

TABLE II

Labeling Distribution in Fatty Acids of *Mesodesma mactroides* after Administration of 1-^{14}C Palmitic and 1-^{14}C Stearic Acids Dissolved in Seawater

Fatty acids	16:0 1-^{14}C			18:0 1-^{14}C		
	3 hr[a]	6 hr	12 hr	3 hr	6 hr	12 hr
14:0	–	1.1 ± 0.6	0.7 ± 0.3	–	–	0.8 ± 0.4
16:0	97.3 ± 1.1[b]	96.2 ± 0.4	94.3 ± 0.8	–	–	–
18:0	2.7 ± 1.1	2.1 ± 0.4	2.1 ± 0.4	100.0 ± 0	93.0 ± 1.2	80.7 ± 0.7
18:1	–	–	2.9 ± 0.5	–	2.7 ± 0.3	12.2 ± 2.0
18:2	–	–	–	–	4.3 ± 1.3	6.3 ± 1.0
Total incorporation %	5.9 ± 1.1[c]	7.0 ± 0.9	14.1 ± 1.3	6.1 ± 1.5	9.2 ± 1.2	14.9 ± 2.5

[a]Administration time in Medium No. 1.

[b]Results expressed as percent of total recovered ^{14}C are the mean of the analysis of three groups of two clams each ± SEM. The chromatograph was run until the 22:6 n-3 peak eluted.

[c]The radioactivity measured in the medium before incubation was taken as 100%.

measured on the gas liquid chromatographic trace.

The lipids were separated into polar and nonpolar classes by absorption on silicic acid and fractionated by thin layer chromatography (TLC) by the procedure described by Moreno et al. (1) and counted.

RESULTS AND DISCUSSION

Incorporation of Labeled Saturated Acids Dissolved in Seawater

In the second paper of this series (2), it was demonstrated that labeled linoleic and α-linolenic acids dissolved in the seawater are incorporated and metabolized by *M. mactroides*. Testerman (3) has also shown that the polychaete *Stauronereis rudolphi* absorbs dissolved fatty acids. A similar absorption of 1-^{14}C palmitic and 1-^{14}C stearic acids dissolved in the seawater is shown in Table I. The distribution of ^{14}C labeling among the different components of the clam after 12 hr incubation demonstrates that both acids are mainly incorporated into lipids and only a small proportion is β-oxidized and expired as CO_2. When these results are compared to the data corresponding to 1-^{14}C linoleic and 1-^{14}C α-linolenic acids experiment (2), it is apparent that both saturated acids are less incorporated and metabolized than were the polyunsaturated acids. These results would suggest, at first instance, that the clam when filtering the sea water absorbs the polyunsaturated acids preferentially to the saturated acids. This conclusion is also apparent when single acids are tested. It is pertinent to remark that in the first paper of these series (1), it was shown that the yellow clam, feeding in its natural habitat incorporated the fatty acids 20:5 n-3 and 22:6 n-3 rather selectively.

Biosynthesis of Fatty Acids from Palmitic and Stearic Acids

Table II shows that the labeled palmitic and stearic acids dissolved in seawater are not only incorporated in the lipids and β-oxidized but also are converted to higher homologs. This conversion depends on time. It produces sequentially different elements of the biosynthetic chain. Palmitic acid is not desaturated to palmitoleic acid in detectable amounts but

TABLE III

Labeling Distribution in the Fatty Acids of the Clam after Administration of 1-^{14}C Palmitic and 1-^{14}C Stearic Acids Dissolved in Seawater in the Presence of Casein Hydrolysate[a]

Fatty acids	16:0 1-^{14}C		16:0 1-^{14}C + protein		18:0 1-^{14}C		18:0 1-^{14}C + protein	
	Percent[a]	RSA[b]	Percent	RSA	Percent	RSA	Percent	RSA
14:0	3.8 ± 1.0	10 ± 2	2.0 ± 0.2	20 ± 1	3.0 ± 0.1	26 ± 4	2.2 ± 0.2	37 ± 3
16:0	83.6 ± 1.7	55 ± 4	87.5 ± 2.1	110 ± 4	—	—	—	—
18:0	4.6 ± 0.5	15 ± 0	4.5 ± 0.4	17 ± 2	86.1 ± 1.8	215 ± 15	90.7 ± 0.5	452 ± 10
18:1	8.0 ± 0.4	28 ± 0	6.0 ± 1.5	30 ± 2				—
18:2	—	—	—	—	2.3 ± 0.7	68 ± 12	1.2 ± 0.3	75 ± 4
20:1	—	—	—	—	6.1 ± 0.8	169 ± 23	4.1 ± 0.7	127 ± 8
20:2	—	—	—	—	2.5 ± 4	87 ± 20	1.8 ± 0.1	90 ± 2

[a]Results are the mean of two pools of four groups of two clams each fed during 12 hr ± extreme deviation of the mean.
[b]Relative specific activity (RSA) = dpm/area of fatty acid in the chromatogram.

instead, it is probably first elongated to stearic acid and then this acid would be desaturated to oleic acid. The undetectable conversion of palmitic to palmitoleic acid is consistent with a similar result found in the land mollusc, *Arion ater* (4).

Since oleic acid is formed, administered stearic acid could also be a substrate for a Δ9 desaturation. The oleic acid would be desaturated to 18:2 n-9 by a Δ6 desaturase since Δ6 desaturase activity has been recognized in the yellow clam (2).

In a second experiment (Table III) in which the yellow clams were maintained in the medium containing 1-^{14}C stearic acid for 12 hr, it was also possible to detect the formation of labeled 20:1 n-9 and 20:2 acids. However, it is important to point out that in marine invertebrates the existence of more than one 20:2 acid belonging to oleic acid family has recently been recognized (5-8). It has been shown that there exists a 20:2 $\Delta^{5,11}$ acid besides the normal product 20:2 $\Delta^{8,11}$. Both acids appear very close in a gas liquid chromatography run. Since the exact structure of the labeled 20:2 acid peak synthesized by *M. mactroides* has not been elucidated, it may be possible that it is constituted of a mixture of isomers. The 20:2 $\Delta^{8,11}$ is generally admitted to derive from 18:2 n-9 acid by elongation, whereas 20:2 $\Delta^{5,11}$ derives from 20:1 n-9 acid by Δ5 desaturation (9). The conversion of 20:1 n-9 to 20:2 $\Delta^{8,11}$ is very improbable since it would require a Δ8 desaturase and the existence of this enzyme has been questioned in some animals (9,10). Therefore, the following sequence of biosynthetic reactions may occur in *M. mactroides*, starting from palmitic and stearic acids.

$$16{:}0 \rightarrow 18{:}0 \rightarrow 18{:}1\ \text{n-9} \rightarrow 18{:}2\ \text{n-9}$$
$$\downarrow \qquad \searrow$$
$$20{:}1\ \text{n-9} \rightarrow 20{:}2\ (\Delta^{5,11})\ \ 20{:}2\ (\Delta^{8,11})$$

However, it is still necessary to confirm the structure of 20:2 acid or acids to be sure of the existence of both pathway.

Since small amounts of label were detected in myristic acid, it very probably corresponds to labeled acetate produced by oxidation of the C^{14} substrates and introduced during de novo synthesis of myristic acid.

Addition of casein hydrolysate to the seawater enhances the Δ6 desaturation activity of the clam and increased amounts of linoleic and α-linolenic acids are converted to γ-linolenic and octadeca-6,9,12,15-tetraenoic acid, respectively (2). No effect on the elongation of linoleic and α-linolenic acid was found. The same experiment performed with palmitic and

TABLE IV

Labeling Distribution in the Fatty Acids of the Clam after Administration of 1-^{14}C Acetate[a]

Fatty acids	Time of administration (hr)			
	6	12	24	48
X_1	0.9 ± 0.4	0.2 ± 0.2	0.4 ± 0.4	0.3 ± 0.2
12:0	1.3 ± 0.1	1.8 ± 0.7	1.1 ± 0.4	0.8 ± 0.0
X_2	5.7 ± 0.2	4.3 ± 0.4	4.1 ± 0.2	2.9 ± 0.4
14:0	33.7 ± 1.6	32.7 ± 1.1	24.6 ± 1.6	12.4 ± 1.8
14:1	6.7 ± 0.1	1.8 ± 0.3	1.7 ± 0.3	0.8 ± 0.2
15:0	–	0.3 ± 0.2	0.8 ± 0.3	0.5 ± 0.1
16:0	12.6 ± 0.4	12.6 ± 0.6	10.8 ± 0.4	13.7 ±1.4
16:1	18.0 ± 0.8	17.5 ± 0.6	17.4 ± 0.3	21.8 ± 2.6
16:2	–	2.6 ± 0.8	0.7 ± 0.3	1.9 ± 0.3
18:0	3.5 ± 0.8	5.6 ± 0.2	4.2 ± 0.4	5.4 ± 1.0
18:1	11.4 ± 0.8	14.5 ± 0.9	22.0 ± 0.5	22.0 ± 1.2
18:2	–	1.2 ± 0.6	2.3 ± 0.8	2.3 ± 0.2
20:1	4.4 ± 0.3	3.7 ± 1.6	6.7 ± 1.1	10.5 ± 0.8
20:2	1.8 ± 0.1	1.2 ± 0.8	2.6 ± 0.4	3.0 ± 0.1
20:3	–	–	0.6 ± 0.2	1.7 ± 0.2

[a]Results expressed as percent of total recovered ^{14}C are the mean of two groups of two clams each ± extreme deviation of the mean. The chromatograph was run until the 22:6 n-3 peak eluted.

stearic acid added to the seawater does not show increased desaturation or elongation (Table III). This result agrees with the effect on Δ9 desaturation found in rats (11-13). In these animals, it was shown that hyperproteic diet (>35% protein) enhances Δ6 desaturation activity but does not modify Δ9 desaturation or elongation reactions. Therefore, these protein effects would confirm previous results.

De novo Biosynthesis of Fatty Acids from Acetate

The available information about the biosynthesis of fatty acids from acetate in aquatic molluscs is very scanty. Shieh (14) injected labeled sodium acetate to the scallop, *Placopecten magellanicus,* and found no radioactivity inthe fatty acids of the phospholipids. From this result, he suggested that the fatty acids of the molluscs were toally provided by the food. In Table IV, it is shown that labeled acetate dissolved in the seawater is absorbed by the yellow clam and converted to different fatty acids of 12, 14, 15, 16, 18, and 20 carbons.

The distribution of the radioactivity among the fatty acids of the clam changed with time of administration. After the first 6 hr, it was very high in myristic acid and the order of the radioactivity of the saturated fatty acids was 14:0 > 16:0 > 18:0. It changed gradually with the elapsed time and after 48 hr, the radioactivity of myristic acid decreased to nearly a third of its original value whereas palmitic acid remained practically constant and stearic acid increased.

The monoethylenic acids 14:1, 16:1, 18:1, and 20:1 were also synthesized from acetate. The distribution of the radioactivity in these also changes with time. The labeling of 14:1 acid was high at the beginning and decreased from 6 to 48 hr whereas the radioactivity of 16:1, 18:1, and 20:1 acids increased in the meantime. From 18:0% to 21.8% of total radioactivity found in the fatty acids after 1-^{14}C acetate administration was detected in 16:1 acid. These data are completely different from the results shown in Tables II and III in which an absence of 1-^{14}C palmitic acid conversion to palmitolaic acid was found when 1-^{14}C palmitate was administered. This difference is difficult to explain but could be related to better availability of the palmitate to the desaturating enzyme when palmitate was endogenously synthesized from acetate. However, the existence of an alternative synthesis of 16:1 from acetate, different from the Δ9 desaturation of palmitic acid, should not be completely disregarded. This mechanism could for example be, the one proposed by Raju and Reiser (15).

The de novo biosynthesis of 18:1, 20:1, 20:2, and 20:3 is also shown in Table IV and the labeling of the acids also increased with time of 1-^{14}C acetate administration.

The time effect found for the three types of acids suggests that higher homologs are synthesized gradually from lower homologs, and Δ9, Δ6, and Δ5 desaturases are present in the yellow clam.

TABLE V

Distribution of Radioactivity in the Lipids of *Mesodesma mactroides* after Administration of 1-^{14}C Palmitic and 1-^{14}C Stearic Acids[a]

Lipid fraction[b]	1-^{14}C 16:0		1-^{14}C 18:0	
	Percent	dpm/mg	Percent	dpm/mg
Total nonpolar lipids	95.0 ± 0.5	2110 ± 205	97.1 ± 1.1	2662 ± 326
Origin zone	27.2 ± 1.3	4408 ± 233	31.6 ± 2.0	6762 ± 1359
Diglycerides	18.2 ± 1.0	2588 ± 381	21.8 ± 1.0	3768 ± 563
Free acids	43.4 ± 0.6	8850 ± 629	38.2 ± 0.1	10182 ± 1407
Triglycerides	4.8 ± 0.3	420 ± 64	3.9 ± 1.9	402 ± 166
Alkoxydiglycerides	1.4 ± 0.3	196 ± 2	1.7 ± 0.7	215 ± 1
Total polar lipids	5.0 ± 0.5	208 ± 38	2.9 ± 1.1	145 ± 19
Origin zone	2.7 ± 0.4	—	1.7 ± 0.5	—
Free acids	2.3 ± 0.2	—	1.2 ± 0.5	—

[a]Results are the mean of two pools of four groups of two clams each fed during 12 hr ± extreme deviations fof the mean.

[b]Lipids were separated into polar and nonpolar classes and fractionated by thin layer chromatograph as described previously (2).

Esterification of Fatty Acids in Lipid Fractions

Table V shows that *M. mactroides* partially esterified absorbed 1-^{14}C palmitic and 1-^{14}C stearic acid. After 12 hr administration of the labeled acids, 45.9% of palmitic acid and 39.4% of stearic acid still remained as free acids. In spite of that, an important amount of labeling was found at the origin of the TLC and was not identified. The radioactivity was mainly found in the diacylglycerol more and less in the triacylglycerol and alkoxyglycerol zone. No labeling was detected in the phospholpids separated by absorption on silicic acid and TLC. Therefore, the incorporation pattern of labeled palmitic and stearic acids, compared to linoleic and α-linolenic acids (2), shows a remarkable preference of the saturated acids for the neutral lipids. In addition, the relatively fast and high labeling of diacylglycerol compared to triacylglycerol suggests that lipid biosynthesis in the yellow clam follows Kennedy's route. This was also shown for linoleic and α-linolenic acids in our preceding work (2).

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The *in vivo* Incorporation of [^{32}P]-labeled Orthophosphate into Pyrophosphatidic Acid and Other Phospholipids of *Cryptococcus neoformans* Through Cell Growth

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ABSTRACT

Cryptococcus neoformans was cultured in a liquid medium containing [^{32}P]-orthophosphate and harvested at various stages of cell growth. An aliquot of the [^{32}P]-labeled cells was transferred to a nonradioactive medium, and the culture was continued again for some hours. The [^{32}P]-radioactivity composition and the phosphorus composition of individual phospholipids relative to the total phospholipid through the incubation periods were estimated. Although levels of major phospholipids remained constant throughout the cell growth, the distribution pattern of the [^{32}P]-radioactivity of individual phospholipids changed remarkably along with the progress of cell growth. The changing patterns of the specific radioactivities of individual phospholipids through the growth phase demonstrated that phosphatidic acid was one of the most active metabolites in phospholipids and that pyrophosphatidic acid was also metabolically active.

INTRODUCTION

A novel phospholipid, pyrophosphatidic acid [*P,P'-bis*-(1,2-diacyl-*sn*-glycero-3-)pyrophosphate], was first isolated and identified in the cellular lipid of yeast (*Cryptococcus neoformans*) (1). Recent work on the species variation of yeast lipids showed that several yeast strains other than *Cr. neoformans* also contained small amounts of pyrophosphatidic acid (2,3). No detectable amount of this lipid, however, was found in the lipid extracts of other natural sources. The positional specificity of the fatty acid in this new lipid coincided with those of ordinary glycerophosphatides (4). The changing profiles of the fatty acid composition of pyrophosphatidic acid through the growth phase of *Cr. neoformans* closely resembled that of phosphatidic acid (5). This evidence suggests that pyrophosphatidic acid might be intimately related to phosphatidic acid, which is situated at a central place in glycerolipid metabolism (6). The purpose of this series of studies was to find the metabolic pathway and the biological function of pyrophosphatidic acid. The present report describes the uptake and turnover of [^{32}P]-radioactivity into pyrophosphatidic acid and other cellular phospholipids of *Cr. neoformans* from a cultural medium containing [^{32}P]-orthophosphate.

[1] Presented in part at the annual meeting of the Agricultural Chemical Society of Japan (Kyoto, 1976).

MATERIALS AND METHODS

Culture of Organism

The organism used was *Cr. neoformans*, strain CBS-132, supplied by Dr. S. Gotoh (Faculty of Engineering, Yamanashi University). The organism was cultured in the following liquid medium: glucose, 100 g; yeast ex-

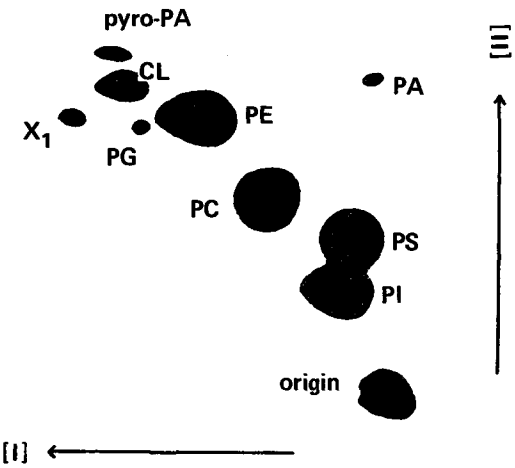

FIG. 1. A typical autoradiogram of [^{32}P]-labeled phospholipids of *Cryptococcus neoformans* at early logarithmic phase (5 hr). Plate: silica gel precoated plate (Merck, 10 x 10 cm). Solvent systems: [I], chloroform:methanol:7N·NH_4OH (65:35:5, v/v); [II], chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, v/v). Abbreviations: pyro-PA, pyrophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; CL, cardiolipin; PG, phosphatidylglycerol; X_1, unidentified phospholipid; origin, perhaps a derivative of the mannosylinositol phosphorylceramide complex or of inositol phosphorylceramide.

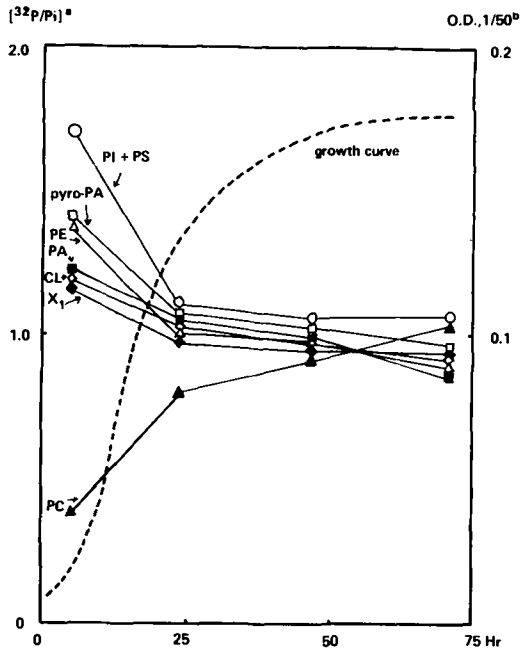

FIG. 2. The growth curve of *Cryptococcus neoformans* and the specific radioactivites of individual phospholipids through the growth phase. Abbreviations: see Figure 1. (a) [32P/Pi] illustrates the ratios of percentages of [32P] radioactivity to percentages of lipid phosphorus of individual phospholipids in total phospholipid. (b) Optical density x 1/50 of the yeast culture. (- - - -); growth curve, (○——○); PS + PI, (□——□); pyro-PA, (■——■); PA, (▲——▲); PC, (◇——◇); CL, (◆——◆); X_1 (unidentified phospholipid); PE, (△——△).

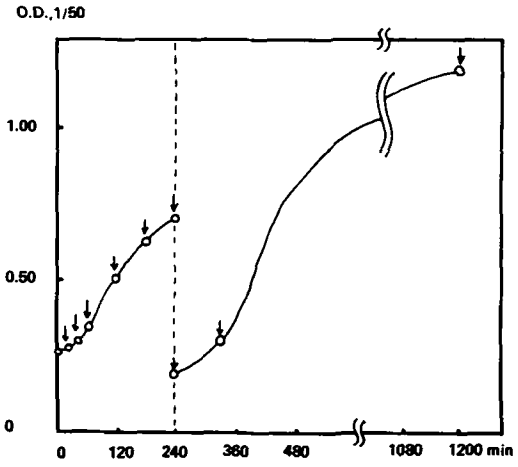

FIG. 3. A growth curve of the short-term culture of *Cryptococcus neoformans*. After 240 min of incubation in [32P]-radioactive medium, a part of the yeast cells was washed rapidly free of [32P] and reincubated in nonradioactive medium for 960 min. Yeast cells were harvested at the times marked with arrows.

tract, 1 g; KH_2PO_4, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; $CaCO_3$, 0.01 g; KCl, 0.28 g; NaCl, 0.01 g; [32P]-Na_3PO_4, 2 mCi (200 mCi/mmol); and distilled water, 1000 ml. The pH of the medium was adjusted to 6.0. Culturing conditions were described in the previous report (5). In the short-term culture of yeast, cells were harvested in the early stages of the growth phase at 15-60 min intervals, and an aliquot of the [32P]-labeled cells was reincubated in nonradioactive medium.

Extraction of lipids and analysis of lipid phosphorus were described in the previous report (5,7).

Thin Layer Chromatography and Autoradiography

Lipids were chromatographed on silica gel precoated plates (Merck). Solvent systems and detecting reagents were as described previously (8). Detection of [32P]-radioactive phospholipids on the chromatograms was achieved by autoradiography with the use of X-ray films (Sakura QH).

Measurement of Radioactivity in Labeled Yeast Cells and Phospholipids

An aliquot (about 1 ml, 10^4 cpm) of the culture was transferred to a membrane filter (Sartorius MF-100) and washed with distilled water. The filtrate and residual cells on the membrane were dried under an infrared lamp. The [32P]-radioactivity of cells and filtrate was counted by an end window Geiger-Müller counter (Aroka). A portion of the total cellular lipids (about 2 mg, 10^4 cpm) was separated on silica gel precoated plates by two-dimensional thin layer chromatography. Spots of radioactive compound on chromatograms were scraped off and counted with the same GM-counter.

Chemicals

The organic solvents were of reagent grade and were freshly distilled before use. Other organic and inorganic reagents, of analytical grade or of the highest quality commercially available, were used without further purification.

RESULTS AND DISCUSSION

Cr. neoformans was cultured for 72 hr in a liquid medium containing [32P]-orthophosphate. Yeast cells were harvested in the early logarithmic phase (5 hr), the late logarithmic phase (23 hr), the early stationary phase (47 hr), and the late stationary phase (71 hr). A typical autoradiogram of major components of the phospholipids of yeast cells harvested at the early logarithmic phase, is shown in Figure 1.

The major phospholipids were identified as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, and cardiolipin, followed by the next group: pyrophosphatidic acid, phosphatidic acid, and an unidentified phospholipid (X_1) which has been tentatively identified as *N,N*-dimethylphosphatidylethanolamine. The spot remaining at the origin was considered likely to be a derivative of the mannosylinositol phosphorylceramide complex or of inositol phosphorylceramides as described in previous reports (3,9). There was essentially no qualitative alteration of the phospholipid composition during the cell growth cycle. Although levels of the major phospholipids remained constant throughout cell growth (5), the distribution pattern of the [^{32}P]-radioactivity of the individual phospholipids changed remarkably with the progress of cell growth. The growth curve of *Cr. neoformans* and the ratio of [^{32}P]-radioactivity (%) to the lipid phosphorus content (%) of each phospholipid at the respective incubation periods are illustrated in Figure 2. In the early logarithmic phase (5 hr incubation), the ratio of [^{32}P] (%)/Pi (%) of phosphatidylserine plus phosphatidylinositol was the highest in phospholipids, followed by pyrophosphatidic acid, phosphatidylethanolamine, cardiolipin, and X_1. On the other hand, the uptake of [^{32}P] into phosphatidylcholine was extremely low at the logarithmic phase. The incorporation of [^{32}P] into individual phospholipids converged to constant levels with the progress of the growth phase. In order to determine the amount of [^{32}P] incorporated into phospholipids in the earlier stages of the cell growth, the yeast cells were cultured in a [^{32}P]-radioactive medium for a short time and harvested at 15-60 min intervals. A portion of the [^{32}P]-labeled cells was transferred to a nonradioactive medium, and the culture was continued for 960 min. A growth curve of the short-term culture of the organism is shown in Figure 3. The overall uptake of [^{32}P]-orthophosphate into yeast cells for 240 min incubation was 30% of the total added [^{32}P]. More than 90% of the incorporated [^{32}P] remained in the cells for 960 min postincubation in the nonradioactive medium. The composition of individual phospholipids throughout the cell growth are summarized in Table I. Only small changes in the amounts of individual phospholipids were found throughout the cell growth. The percentage of [^{32}P]-radioactivity of each phospholipid is presented in Table II. Significant amounts of [^{32}P]-radioactivity were detected in the phosphatidylserine and phosphatidylcholine fraction of the cells harvested at 15 min incubation, and

TABLE I

Percent of Lipid Phosphorus of Individual Phospholipids of *Cryptococcus neoformans*[a]

Phospholipids	Incubation time (min)							
	15	30	60	120	180	240	330	1200
pyro-PA	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.2	0.7 ± 0.1
PA	1.0 ± 0.1	0.9 ± 0.1	1.3 ± 0.1	1.4 ± 0.2	1.3 ± 0.1	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1
CL	5.4 ± 0.2	6.0 ± 0.2	5.5 ± 0.1	5.5 ± 0.2	5.6 ± 0.1	5.8 ± 0.2	5.5 ± 0.2	5.4 ± 0.2
PS	12.5 ± 0.5	13.0 ± 0.5	13.0 ± 0.5	13.0 ± 0.5	13.0 ± 1.0	13.0 ± 0.5	12.5 ± 0.7	12.0 ± 0.5
PI	8.0 ± 1.0	7.5 ± 0.8	8.4 ± 0.6	8.5 ± 1.5	8.0 ± 1.0	7.0 ± 1.0	6.5 ± 0.5	7.0 ± 1.0
PE	20.0 ± 2.0	20.5 ± 1.0	21.0 ± 1.0	21.0 ± 1.0	22.0 ± 1.5	22.5 ± 1.5	21.5 ± 1.0	22.5 ± 1.0
PC	48.0 ± 2.5	46.0 ± 2.0	45.0 ± 1.5	45.0 ± 2.0	45.0 ± 1.5	44.5 ± 1.5	46.0 ± 2.0	46.5 ± 2.0
X_1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.2	0.4 ± 0.2	0.6 ± 0.1	0.7 ± 0.2	0.4 ± 0.1	0.6 ± 0.2
origin	4.0 ± 0.5	4.5 ± 1.5	4.0 ± 0.5	4.5 ± 1.0	4.0 ± 1.5	4.5 ± 1.0	4.5 ± 1.0	4.0 ± 1.0

[a]After 240 min of incubation in [^{32}P]-radioactive medium, a part of the yeast cells was reincubated in nonradioactive medium for 960 min. The figures are the means ± SD of the values obtained from three repetitions of lipid phosphorus determination by Bartlett (11). Abbreviations: see Figure 1.

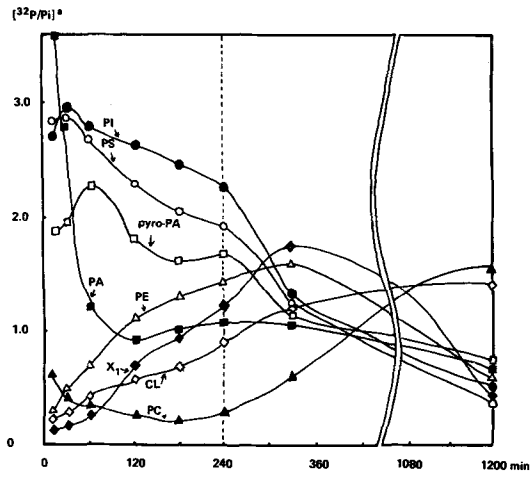

FIG. 4. The specific radioactivities of individual phospholipids through the growth phase of short-term culture. (a) [^{32}P/Pi] illustrates the ratios of percentages of [^{32}P] radioactivity to percentages of lipid phosphorus of individual phospholipids in total phospholipid. Abbreviations: see Figure 1.

the remarkable changes of [^{32}P]-distribution patterns in phospholipids were observed through the growth stages. The ratio of [^{32}P] (%)/Pi (%) of each phospholipid in short-term incubation is illustrated in Figure 4. Since the ratio of [^{32}P]-radioactivity to lipid phosphorus was much higher in phosphatidic acid than in the other phospholipids in extremely early stages of culture, it seemed likely that phosphatidic acid might be a key substance in the biosynthesis of glycerolipid in *Cr. neoformans*, as found in the other yeast species (10). Although a significant amount of radioactivity was incorporated into phosphatidylcholine at early stages of growth (15 min), the specific radioactivity of phosphatidylcholine was remarkably low and decreased with the progress of growth stages. Perhaps it would suggest that a part of phosphatidylcholine turnover might be more rapid than the others. The specific activity of phosphatidylserine and phosphatidylinositol were maximum at 30 min and decreased with growth stages, and of pyrophosphatidic acid being maximum at 60 min and decreasing with growth. These appearances might support the possibility that phosphatidic acid, phosphatidylserine, phosphatidylinositol, or phosphatidylcholine would be biosynthetic precursors of pyrophosphatidic acid. On the other hand, it had been proposed in a previous report that the changing profiles of the fatty acid composition of pyrophosphatidic acid through the growth phase of *Cr. neoformans* closely resembled that of phosphatidic acid and did not resemble those of phosphatidylcho-

TABLE II

Percent of [^{32}P]-Radioactivities of Individual Phospholipids of *Cryptococcus neoformans*[a]

Phospholipids	Incubation time (min)							
	15	30	60	120	180	240	330	1200
pyro-PA	1.3 ± 0.2	1.6 ± 0.2	1.5 ± 0.1	1.4 ± 0.2	1.2 ± 0.1	1.4 ± 0.2	0.7 ± 0.1	0.5 ± 0.1
PA	4.4 ± 0.1	2.4 ± 0.1	1.5 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	1.0 ± 0.1	1.0 ± 0.1	0.7 ± 0.2
CL	1.3 ± 0.1	2.0 ± 0.2	2.3 ± 0.2	3.5 ± 0.2	4.5 ± 0.1	5.2 ± 0.2	6.4 ± 0.1	7.2 ± 0.1
PS	38.2 ± 2.0	37.0 ± 2.0	34.0 ± 1.0	28.0 ± 2.0	27.0 ± 1.0	26.0 ± 2.0	15.0 ± 0.5	4.0 ± 1.0
PI	16.0 ± 1.0	23.0 ± 2.0	23.0 ± 2.0	23.0 ± 2.0	18.0 ± 1.0	14.0 ± 2.0	8.0 ± 0.5	3.5 ± 0.2
PE	5.0 ± 0.5	10.5 ± 1.0	16.0 ± 1.0	23.0 ± 1.0	28.0 ± 1.0	32.0 ± 1.0	35.0 ± 1.0	12.0 ± 0.5
PC	31.0 ± 1.0	20.0 ± 2.0	17.0 ± 3.0	14.0 ± 3.0	12.0 ± 3.0	14.0 ± 2.0	28.5 ± 2.0	68.0 ± 2.0
X_1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.3 ± 0.1	0.6 ± 0.1	1.2 ± 0.1	0.4 ± 0.2	0.2 ± 0.1
origin	2.0 ± 0.5	3.5 ± 0.5	4.5 ± 0.5	6.0 ± 1.5	7.0 ± 2.0	5.0 ± 2.0	5.5 ± 1.5	2.5 ± 0.5

[a]The figures are obtained from the same experiments in Table I. Abbreviations: see Figure 1.

line, phosphatidylserine, or phosphatidylinositol, pyrophosphatidic acid might be metabolically closely related to phosphatidic acid (5). It seemed likely that the results of [^{32}P]-incorporation into these lipids were consistent with the consideration in the previous report. The ratio of [^{32}P] (%)/Pi (%) of phosphatidylcholine at 15 min incubation was slightly higher than that of phosphatidylethanolamine, X_1 or cardiolipin. The first slowly decreased, and the other increased with the progress of cell growth. In the nonradioactive medium, the [^{32}P] (%)/Pi (%) ratio was high in phosphatidylethanolamine and X_1 (1.8-1.9) at 90 min postincubation, but it was decreased to the level of 0.4-0.7 in 960 min. On the other hand, significant amounts of [^{32}P]-radioactivity accumulated in the phosphatidylcholine and cardiolipin fractions at 960 min postincubation in the nonradioactive medium. These results demonstrate that the phosphate residues of phosphatidylcholine and cardiolipin are metabolically more retarded than those of the other phospholipids. The composition of fatty acid and its changing pattern of phosphatidylethanolamine closely resembled that of X_1 and phosphatidylcholine throughout the growth phase (5). In the previous report, it was suggested that there is a possibility of stepwise methylation of phosphatidylethanolamine to give phosphatidylcholine in the cells of *Cr. neoformans*.

The in vitro investigation of the metabolism and biosynthesis of pyrophosphatidic acid is being carried out with the use of [^{14}C]- and [^{32}P]-labeled pyrophosphatidic acid, phosphatidic acid, CDP-diglyceride, and glycerophosphate. In the next paper, the degradation process of pyrophosphatidic acid will be described.

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Inhibition by 5-(Tetradecyloxy)-2-Furoic Acid of Fatty Acid and Cholesterol Synthesis in Isolated Rat Hepatocytes

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ABSTRACT

Fatty acid and cholesterol synthesis in isolated rat hepatocytes were strongly inhibited by 5-(tetradecyloxy)-2-furoic acid. With either ^3H_2O or [2-^{14}C]acetate as the labeled precursor, the concentrations of inhibitor causing 50% decrease in fatty acid and cholesterol synthesis were, respectively, <0.005 mM and 0.020 mM. At 0.1 mM inhibitor, citrate concentration in cells from fed rats was increased by 75%; lactate and pyruvate concentrations were decreased by 30%; ethanol oxidation was decreased by 20%; with cells from starved rats, the mitochondrial [NAD^+]/[NADH] was decreased. Other parameters were unaffected. Both its potency and its specificity indicate that 5-(tetradecyloxy)-2-furoic acid will be useful in studies on the regulation of lipid biosynthesis.

INTRODUCTION

It was recently reported that feeding TOFA [5-(tetradecyloxy)-2-furoic acid or RMI 14,514, a product of Merrell-National Laboratories] to rats for 4 days decreases the rate of [U-^{14}C]alanine incorporation into hepatic fatty acids in vivo (1). Other preliminary reports (2,3) also indicate that dietary TOFA lowers blood lipids in rats and monkeys and decreases hepatic synthesis of fatty acids. From those reports, however, it is not possible to decide whether TOFA acts by inhibiting reactions involved in lipid biosynthesis or by lowering the enzymatic capacity for lipid synthesis (e.g., decreasing hepatic enzyme concentrations). It has been shown with isolated mitochondria that TOFA inhibits tricarboxylate anion translocation (1). An inhibitor which blocks citrate transport would be useful in studying mitrochondrial-cytosolic interactions and the regulation of lipid synthesis. Therefore, we have tested the effects of TOFA on isolated hepatocytes and have found that it is a potent inhibitor of fatty acid and cholesterol synthesis but has little or no effect on a variety of related metabolic parameters.

MATERIALS AND METHODS

Male Wistar rats (170-220 g body wt) were obtained from Carworth (Wilmington, MA) and were maintained on NIH standard rat chow (5.5% fat, 23.5% protein, 54.5% carbohydrate).

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TABLE I

Effect of TOFA on Rates of Fatty Acid and Cholesterol Synthesis[a]

	Fatty acid synthesis		Cholesterol synthesis	
	^3H_2O	[^{14}C]acetate	^3H_2O	[^{14}C]acetate
Control	0.105±0.019	0.089±0.032	0.019±0.004	0.010±0.001
	Percent decrease from control			
[TOFA] mM				
0.005	56.1 ± 4.1	69.2 ± 14.5	21.0 ± 6.5	26.5 ± 10.7
0.010	77.4 ± 1.7	87.9 ± 6.7	28.8 ± 7.1	36.8 ± 13.3
0.020	86.0 ± 3.3	96.4 ± 2.3	48.5 ± 7.3	52.6 ± 20.3
0.050	89.6 ± 5.2		66.9 ± 5.5	
0.100	89.9 ± 5.4		75.9 ± 5.5	

[a] The control rates are μmol tritiated water or acetate incorporated/min/g wet wt of cells. All values are means ± SEM for three to six cell preparations. Where [^{14}C]acetate incorporation was measured, the incubations contained 10 mM acetate initially. Other conditions and procedures are described in Materials and Methods.

For experiments on fatty acid and cholesterol synthesis, animals were meal-fed between 9 and 12 a.m. for 2 weeks prior to use. Other animals were either fed ad libitum or starved for 48 hr.

Isolated hepatocytes were prepared by the procedure of Berry and Friend (4), modified as described previously (5,6) except that, to minimize glycogenolysis, 20 mM glucose was added to the perfusion medium for rats that were meal-fed or fed ad libitum (7). Incubations of hepatocytes were conducted in 25 ml Erlenmeyer flasks at 37 C as described elsewhere (5,6) except, where rates of fatty acid and cholesterol synthesis were measured, ca. 100 mg wet weight of cells were incubated in 2 ml of medium containing either 1 mCi of ^3H_2O or 1 μCi of [1-^{14}C]acetate. These incubations were stopped by adding 0.5 ml of 10 N NaOH followed by shaking at 37 C for 30 min. Then the contents of the flasks were transferred to screw-cap culture tubes, the flasks washed with 1 ml of water, and this wash combined with the sample. Thereafter, the procedure described by Brunengraber et al. (8) for determination of fatty acids and cholesterol was followed. All incubations were for 60 min and where rates are reported, they are average rates for the entire incubation period.

Metabolites were measured enzymatically: citrate by the method of Dagley (9), ethanol as described by Dickinson and Dalziel (10), and other metabolites as reported previously (11,12). Enzymes and cofactors were obtained from Boehringer Mannheim (Indianapolis, IN). Bovine serum albumin, fraction V, was a product of Miles Laboratories (Elkhart, IN). Tritiated water, 100 mCi/g water and sodium [1-^{14}C]acetate, 2.16 mCi/mmol, were obtained from New England Nuclear (Boston, MA). Other chemicals were reagent grade commercial products.

TOFA or RMI 14,514 was a gift of Dr. Alfred Richardson, Jr. of Merrell-National Laboratories (Cincinnati, OH). Because of the low aqueous solubility of TOFA or its sodium salt, the concentrations used in these experiments were achieved by preparing a stock solution of TOFA in acetone, transferring aliquots to incubation flasks, and evaporating the acetone under a stream of nitrogen. Then 1 ml of incubation medium (Krebs-Henseleit saline containing 2.5% bovine serum albumin) was placed in each flask and shaken at 37 C before adding cells.

TABLE II

Tests for Effects of TOFA on Metabolism of Hepatocytes from Starved Rats[a]

Additions	Metabolite content						Metabolic rates	
	μmol/g wet wt of cells				μmol/ml		μmol/min/g	
	Citrate	ATP	ADP	AMP	Lactate	Pyruvate	Glucose synthesis	Ethanol oxidation
None	0.33 ± 0.06	2.47 ± 0.19	1.01 ± 0.11	0.28 ± 0.03	---	---	0.10 ± 0.01	1.43 ± 0.15
TOFA	0.26 ± 0.09	2.40 ± 0.26	1.07 ± 0.09	0.24 ± 0.03	--	--	0.10 ± 0.01	1.41 ± 0.20
Lactate	0.61 ± 0.09	2.79 ± 0.15	0.84 ± 0.19	0.24 ± 0.06	6.24 ± 0.14	0.60 ± 0.03	0.75 ± 0.05	2.79 ± 0.09
Lactate + TOFA	0.52 ± 0.06	2.40 ± 0.12	0.77 ± 0.19	0.25 ± 0.03	6.48 ± 0.18	0.54 ± 0.04	0.70 ± 0.06	2.35 ± 0.10
Pyruvate	0.56 ± 0.03	2.94 ± 0.15	0.93 ± 0.17	0.20 ± 0.03	1.02 ± 0.18	0.20 ± 0.02	0.84 ± 0.01	---
Pyruvate + TOFA	0.67 ± 0.04	2.91 ± 0.15	0.94 ± 0.15	0.20 ± 0.01	1.14 ± 0.15	0.20 ± 0.02	0.83 ± 0.03	---

[a]Values are means ± SEM for thee to six cell preparations. Initial substrate concentrations were 10 mM for lactate and 5 mM for pyruvate. Where it was added, TOFA concentration was 0.10 mM. No measurements were made in those instances indicated by blank entries. Rates of glucose synthesis were calculated from the total glucose found at the end of a 60-min incubation. The amounts of glucose present at zero time were insignificant and no correction was required. To determine rates of ethanol oxidation, separate incubations were run and contained 8 mM ethanol initially in addition to the indicated substrates.

RESULTS AND DISCUSSION

The data in Table I show that TOFA is a very effective inhibitor of lipid synthesis by iso-

TABLE III

Tests for Effects of TOFA on Ketogenesis and the 3-Hydroxybutyrate/Acetoacetate Ratio[a]

Substrates	(3-Hydroxybutyrate + Acetoacetate) TOFA -	+	3-Hydroxybutyrate / Acetoacetate -	+
Lactate	0.93 ± 0.08	1.00 ± 0.13	0.27 ± 0.03	0.37 ± 0.02
Oleate	7.78 ± 0.43	8.45 ± 0.46	0.40 ± 0.04	0.58 ± 0.03
Lactate, oleate	4.27 ± 0.21	4.72 ± 0.27	0.69 ± 0.05	0.93 ± 0.01
Caproate	15.42 ± 1.47	15.10 ± 2.13	0.47 ± 0.09	0.75 ± 0.20
Lactate, caproate	9.69 ± 0.85	10.71 ± 1.12	0.91 ± 0.22	1.24 ± 0.12

[a]Hepatocytes were from 48 hr starved rats. Values are means ± SEM for three cell preparations. Initial substrate concentrations were 10 mM for lactate, 1 mM for oleate, and 2.5 mM for caproate. Where it was added, TOFA concentration was 0.10 mM. Values for (3-hydroxybutyrate + acetoacetate) are μmol/4 ml incubation containing an average hepatocyte wet wt of 93 mg.

lated hepatocytes. The rate of fatty acid synthesis was decreased more than 50% by 0.005 mM TOFA, but the concentration required to inhibit cholesterol synthesis by 50% was 0.020 mM. TOFA, therefore, is a more potent inhibitor than either clofibrate (ethyl-p-chlorophenoxy-isobutyrate) or (-)hydroxycitrate. Clofibrate inhibits both the fatty acid (13) and the cholesterol (14) synthesizing pathways, but is much less effective than TOFA; i.e., to inhibit fatty acid synthesis in hepatocytes by 50% required 5 mM clofibrate, and 2 mM was needed to give 50% inhibition of cholesterol synthesis (A.C. Sullivan, J. Triscari, G.A. Cook, and J.A. Ontko, unpublished results). Also with hepatocytes, (-)hydroxycitrate, and inhibitor of ATP-citrate lyase (15), caused 50% inhibition of both pathways at ca. 2 mM (7,16).

TOFA also inhibited the incorporation of [1-^{14}C]acetate into fatty acids and cholesterol (Table I), and the pattern of inhibition was the same as when ^3H_2O was the radioactive precursor.

Since fatty acids can affect a variety of metabolic processes, the inhibition of lipogenesis by TOFA (which is structurally similar to a fatty acid) conceivably could be a secondary result. For example, fatty acids can uncouple oxidative phosphorylation by isolated mitochondria (17) and can inactivate purified phosphofructokinase (18). The possibility that TOFA might decrease lipid synthesis by indirect means was tested by examining the effects of TOFA on other metabolic parameters. Some tests were performed with hepatocytes from fed rats; other tests, e.g., ketogenesis from added fatty acids and gluconeogenesis, were facilitated by using cells from starved rats. The general aim of these experiments was to define the specificity of TOFA's action. Any new metabolic inhibitor should be well characterized in this regard, particularly if it is to be used in vivo or with complex systems such as intact cells. The results shown in Tables II-IV were all obtained with 0.1 mM TOFA; lower concentrations of the drug, e.g., 0.010 mM which inhibits fatty acid synthesis by about 80%, had no detectable effect on any of the parameters tested.

TOFA had no significant effect on either the total adenine nucleotide content or the relative amounts of ATP, ADP, and AMP with hepatocytes from fed or starved rats (Tables II and IV). The effect of nutritional state on relative amounts of ATP, ADP, and AMP agrees with that seen by others (19). That TOFA did not affect the cell's ability to meet metabolic energy demands is further indicated by measurements of glucose synthesis. With lactate or pyruvate as the precursor, gluconeogenesis occurred at similar rates in the presence or absence of TOFA (Table II). The same result was obtained with dihydroxyacetone as the glucose precursor.

In view of the report that TOFA blocks tricarboxylate translocation in isolated mitochondria (1), we performed experiments on ethanol oxidation and ketogenesis as tests for actions of TOFA on other mitochondrial transfer processes. Cytosolic NADH produced from ethanol metabolism is reoxidized in part via the mitochondrial malate-asparate shuttle (20). Since neither ethanol oxidation nor gluconeogenesis from lactate or pyruvate (which also involves mitochondrial shuttles) was inhibited (Table II), it appears that the malate-aspartate shuttle is not affected by TOFA. Similarly, as evidenced by ketone body production, TOFA did not inhibit mitochondrial uptake of fatty acids (Table III) either directly (caproate) or via the carnitine-acyl transferase system (oleate). There

was, however, a slight increase in the 3-hydroxybutyrate/acetoacetate ratio indicating that TOFA caused a decrease in the mitochondrial free [NAD^+]/[NADH] ratio (21).

With hepatocytes from fed animals (Table IV), there was no effect of TOFA on glucose production, but small consistent decreases were observed in lactate and pyruvate accumulation and in ethanol oxidation. The former may indicate a slight inhibitory effect of TOFA on glycolysis; no explanation is apparent for the effect on ethanol oxidation. The most significant effect in these experiments was on citrate which increased by 75% in cells incubated with 0.1 M TOFA. With isolated mitochondria, TOFA strongly inhibits tricarboxylate anion translocation (K_I=0.0014 mM; Ref. 1), but, with hepatocytes, citrate levels were unchanged by 0.010 mM TOFA although fatty acid synthesis was decreased by 80%. Our measurements provide no information concerning the distribution of citrate between mitochondria and the cytosol, but a very large gradient would be required to account for the effects of TOFA on fatty acid synthesis.

Since 80% of the cellular acetyl CoA synthetase activity is found in the cytosolic fraction (22), it might be expected that acetate activation would occur primarily in the cytosol. In that view, inhibition of mitochondrial citrate transport should have little or no effect on substrate supply for fatty acid and cholesterol synthesis in hepatocytes incubated with acetate. By this reasoning, we had not anticipated the results in Table I showing the strong inhibitory effect of TOFA on [^{14}C]acetate incorporation into fatty acids and cholesterol. One explanation for these results is that, with intact cells, TOFA acts most strongly on some process other than mitochondrial citrate transport. An alternative explanation is suggested by recent data indicating that coenzyme A concentration in the cytosol is 10 to 60 times lower than in mitochondria (23,24). Those data, the potency with which TOFA inhibits mitochondrial citrate transport, and our results in Table I suggest the possibility that acetate activation to acetyl CoA occurs primarily in mitochondria. Further experiments will be required to test that possibility and to define the processes involved in the inhibition of lipid synthesis by TOFA. The results presented here, however, show that TOFA is a relatively specific inhibitor which will be useful in studies on the regulation of fatty acid and cholesterol synthesis.

TABLE IV

Tests for Effects of TOFA on Metabolism of Hepatocytes from Fed Fats

Additions	Metabolite content						Metabolic rates	
	μmol/g wet wt of cells				μmol/ml		μmol/min/g	
	Citrate	ATP	ADP	AMP	Lactate	Pyruvate	Glucose accumulation	Ethanol oxidation
None	0.34 ± 0.02	2.86 ± 0.12	0.44 ± 0.10	0.10 ± 0.02	1.15 ± 0.13	0.27 ± 0.04	1.49 ± 0.10	1.78 ± 0.04
TOFA	0.60 ± 0.08	3.05 ± 0.13	0.56 ± 0.06	0.10 ± 0.01	0.77 ± 0.09	0.17 ± 0.03	1.51 ± 0.16	1.40 ± 0.11

[a]Hepatocytes were from rats fed ad libitum and were incubated either in the absence or in the presence of 0.1 mM TOFA. Values are means ± SEM for three experiments. Lactate and pyruvate values are concentrations in the incubated cell suspensions. Glucose accumulation is the rate of glucose appearance in the cell suspensions corrected for glucose present at zero time, and it includes glucose production from glycogenolysis and any glucose synthesis from endogenous substrates. For measurement of ethanol oxidation, separate incubations were run and contained initially 8 mM ethanol.

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Arylsulfonate Esters as Hypocholesteremic Agents: III. Mechanism of Action Studies[1]

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ABSTRACT

The mechanism responsible for the hypocholestermic action of arylsulfonate esters of long chain fatty alcohols has been studied with rats fed either normocholesteremic or hypercholesteremic (1% cholesterol plus 0.5% glycocholate) diets. Linoleyl tosylate is more effective in lowering plasma and liver cholesterol levels of rats on the hypercholesteremic diet than several other hypocholesteremic agents tested. Linoleyl tosylate does not redistribute cholesterol to extrahepatic tissues nor inhibit hepatic cholesterol biosynthesis. Linoleyl tosylate is not effective in counteracting Triton-induced hypercholesteremia nor in lowering plasma cholesterol levels of the suckling rat. Linoleyl tosylate increases the fecal elimination of dietary [4-^{14}C] cholesterol and prevents its accumulation in blood and liver. Oleyl p-(n-decyl) benzene sulfonate also prevents the apparent absorption of [26-^{14}C] cholesterol from the gastrointestinal tract. Linoleyl tosylate increases the fecal excretion of neutral sterols but not of bile acids. The results indicate that the arylsulfonate esters of long chain fatty alcohols lower body cholesterol levels by inhibiting cholesterol absorption from the gastrointestinal tract. Exactly how absorption is inhibited is not clear, but linoleyl tosylate was found to stimulate the activity of cholesteryl esterase prepared from the intestinal mucosa.

INTRODUCTION

Arylsulfonate esters of various long chain fatty alcohols have been found effective both in lowering plasma and liver cholesterol levels in hypercholesteremic rats and in controlling levels in normocholesteremic rats placed on hypercholesteremic diets (1,2). The hypocholesteremic action of these compounds has been shown in a number of different species, including cholesterol-fed rabbits, pigeons, mice, and monkeys (2). In light of the greater effectiveness of the arylsulfonate esters in preventing the accumulation of both plasma and liver cholesterol in comparison with all other hypercholesteremic agents tested in the cholesterol-fed rat, the mechanism by which the arylsulfonate esters bring about the hypocholesteremic response has been pursued.

Linoleyl tosylate was chosen for many of the mechanism of action studies because this ester has been used as the reference compound for structural requirements (2). In addition, linoleyl tosylate was the most active of the various arylsulfonate esters which had been synthesized and investigated at the time of most of these studies. The more potent oleyl p-(n-decyl) benzene sulfonate was used in one study and appears to act via the same mechanism as linoleyl tosylate.

METHODS AND MATERIALS

Animals and Diets

Rats of the Wistar strain were used in all experiments. The basic composition of the diets has been described previously (1,2). Diet C was the colony diet; Diet F was a hypercholesteremic, semisynthetic diet 1% in cholesterol and 0.5% in sodium glycocholate; Diet G contained the same components of Diet F except cholesterol and glycocholate and, therefore, was not hypercholesteremic; Diet H was the colony diet rendered hypercholesteremic with 1% cholesterol and 0.5% sodium glycocholate; and Diet I was similar to Diet F but contained 5% hydrogenated coconut oil, 5% bulk (celluflour), 1% cholesterol, and 0.25% sodium glycocholate.

Analysis

Blood samples were obtained by heart

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puncture with heparinized syringes. Plasma and liver free and total cholesterol values were estimated after purification of the digitonides by the Sperry-Webb method (3).

In the biosynthesis studies, total lipids were extracted from liver by a chloroform-methanol procedure (4). Liver sterols were separated from a saponified aliquot of the total lipid extract by digitonin precipitation. The digitonides, washed with 80% ethanol, acetone: ether (2:1,v/v), and ether 2 times, were dissolved in methanol and assayed for radioactivity in toluene scintillation fluid (4 g/l. 2,5 diphenyloxazole and 0.1 g/l. 1,4-bis-2(5-phenyloxazoyl)-benzene) with a Packard Liquid Scintillation counter equipped with differential discriminators for ^{14}C and ^{3}H. Corrections with internal standards were made for quenching throughout these experiments when necessary.

Isotopic steady state studies were performed essentially as described by Wilson (5). Male rats were implanted subcutaneously with gelatin pellets containing ca. 100 mg of [4-^{14}C] cholesterol (50,000 cpm/mg). After 6 weeks equilibration on Diet C (stock diet), the experimental diets were provided. Two weeks later, feces were collected daily for 2 days; the animals were then killed the last day by exsanguination. Feces were extracted with chloroform-methanol and the extract was separated into saponifiable and nonsaponifiable fractions after high pressure saponification (5). Blood and liver cholesterol specific activities were established to determine extent of equilibration. Total radioactivity was assayed on the total fecal extract and the two saponification fractions. Calculations were based upon the equations similar to those given by Wilson (5):

$$\text{mg neutral sterols + bile acid excreted} = \frac{\text{CPM in total fecal extract}}{\text{blood cholesterol SpAc}}$$

$$\text{mg neutral sterols excreted} = \frac{\text{CPM in nonsaponifiable fraction}}{\text{blood cholesterol SpAc}}$$

$$\text{mg bile acids excreted} = \frac{\text{CPM in saponifiable fraction}}{\text{blood cholesterol SpAc}}$$

In studies on the elimination of a fed dose of [4-^{14}C] cholesterol, the radioactivity was extracted by the procedure described by Wilson (5) and assayed for radioactivity as described above.

The amount of label appearing in tissues of rats fed a single meal containing [4-^{14}C] cholesterol was determined by assay of radioactivity in an acetone-ethanol extract (3) of blood or plasma and a hexane-ethanol extract (6) of liver.

Feces were extracted with chloroform-methanol (5) and the extract separated into neutral sterol and bile acid fractions (7). Neutral sterol extracts were analyzed for slow- and fast-acting sterols as described by Moore and Baumann (8). Bile acids were estimated as described by Levin et al. (9).

Digestive tract contents, which were obtained by rinsing the tracts with distilled water, were extracted by refluxing with chloroform: methanol (2:1) and chloroform, each for 1 hr. The carcasses (including digestive tracts) were digested in 30% KOH as described previously (10). An aliquot of the digest was acidified to pH 2 with HCl and extracted with chloroform. Aliquots of extracts were taken to dryness and counted in toluene scintillation fluid. Hyamine hydroxide was used to collect the radioactive CO_2 evolved during acidification of the carcass digest.

Expired CO_2 was collected by placing animals in sealed chambers through which ascarite-scrubbed air (1.25 l/min) was circulated and then bubbled through 2 N KOH in two consecutive 1500 ml traps. Carbonate was precipitated from aliquots of the KOH traps with saturated $BaCl_2$. The precipitate was filtered, washed with water, dried at 110 C, weighed, pulverized, and counted in a toluene scintillation fluid containing 4.5% Thixotripic Gel Powder (Packard Instrument Co., Inc.).

Cholesteryl Esterase (Sterol-ester Hydrolase, EC 3.1.1.13) Activity

Acetone powders of rat intestinal mucosa were prepared by the procedures of Lossow et al. (11) and Shah et al. (12). One ml of a distilled water extract of the acetone powder was added to 3.5 ml of 0.1 M potassium phosphate buffer, pH 6.2, containing 10 mg of sodium taurocholate. [4-C^{14}] cholesterol (5 μmoles) was added in 100 μl of acetone; fatty acid (3.5 μmoles) in 25 μl of acetone; and linoleyl tosylate (10 μmoles in 50 μl of acetone. Each flask contained an equivalent amount (200 μl) of acetone. Incubations were conducted at 37 C for 3 hr. Cholesteryl esters were separated as a group from hexane-ethanol extracts (6) by thin layer chromatography (TLC) (adsorbent, Silica Gel G with rhodamine-6-G indicator; solvent system, hexane:ether:acetic acid, 70:30:1, v/v/v), extracted with ether, and assayed for radioactivity as described above.

Materials

Linoleyl tosylate and oleyl p-(n-decyl) benzene sulfonate were synthesized in this

TABLE I

Comparative Effects of Various Hypocholestermic Compounds on Plasma and Liver Cholesterol Levels of Hypercholestermic Rats[a]

Treatment (%)	Plasma total cholesterol	Liver cholesterol	
		Total	Free
	mg %	mg/g wet wt	
Control	280 ± 30	64 ± 7	3.9 ± 0.2
Thyroxin (0.003)	270 ± 26	52 ± 2	3.6 ± 0.3
CPIB ethyl ester (0.15)	180 ± 15[b]	55 ± 3	3.1 ± 0.2[b]
Benzmalecene (0.15)	190 ± 16[b]	60 ± 3	3.5 ± 0.3
SK & F 525A (0.15)	75 ± 4[b]	75 ± 3	3.0 ± 0.1[b]
Linoleyl tosylate (0.15)	85 ± 4[b]	23 ± 4[b]	2.4 ± 0.1[b]

[a]Weanling male rats were preconditioned for 4 weeks on Diet F-1 (hypercholesteremic diet with 1% hydrogenated coconut oil). They were then given the same diet supplemented with various hypocholesteremic compounds with 4-6 animals per group for 3 weeks.

[b]$P < 0.05$.

laboratory as described previously (2). The preparations were found to be homogeneous by TLC (adsorbent, Silica Gel G; solvent system, hexane:ether:acetic acid, 70:30:1, v/v/v). Other compounds were of commercial origin as follows: cholesterol, glycocholate, digitonin, and L-thyroxine (Nutritional Biochemical Corp., Cleveland OH); hydrogenated coconut oil (contributed by Procter & Gamble, Cincinnati, OH); [1-^{14}C] acetate (Calbiochem, Los Angeles, CA); [5-^{3}H] mevalonate (New England Nuclear Corp., Boston, MA); [4-^{14}C] cholesterol (Tracerlabs, Waltham, MA); [26-^{14}C] cholesterol (Amersham/Searle Corp., Arlington Heights, IL); fatty acids (Hormel Institute, Austin, MN); sodium taurocholate (Sigma Chemical Co., St. Louis, MO); Triton WR-13939 (kindly donated by Rohm and Haas, Inc., Philadelphia, PA); ethyl p-chlorophenoxyisobutyrate (kindly donated by Ayrest Laboratories, Inc., New York, NY); SK & F 525-A (kindly donated by Smith, Kline & French Laboratories, Philadelphia, PA); benzmalecene (kindly donated by Merck Sharp & Dohme Research Laboratories, West Point, PA).

RESULTS

Linoleyl tosylate is more effective than several other hypocholesteremic agents in the cholesterol plus glycocholate-fed rat. The effects of the various hypocholesteremic compounds on blood and liver cholesterol are given in Table I. Linoleyl tosylate was the only compound which lowered both plasma and liver cholesterol levels. SK & F 525A was as effective as linoleyl tosylate in lowering plasma cholesterol levels but was ineffective upon hepatic cholesterol.

Linoleyl tosylate does not redistribute cholesterol to extra-hepatic tissues. Total carcass lipid was extracted from animals which had received linoleyl tosylate for 4 weeks in a therapeutic study (Table II). The total lipid and total cholesterol levels were reduced below levels observed in control, hypercholesteremic animals. Since the noncholesterol fraction of the nonsaponifiable material was a constant, the tosylate action was evidently directed only at cholesterol. The results indicate further, that in lowering body cholesterol, linoleyl tosylate did not induce the accumulation of some other sterol in the tissues.

Linoleyl tosylate is ineffective in counteracting Triton-induced hypercholesteremia and suckling rat hypercholesteremia. The hypercholesteremia induced by Triton is believed to constitute a simple, rapid test system for the preliminary evaluation and classification of potential hypocholesteremic compounds (13). However, linoleyl tosylate was completely ineffective on both the first and second phases of triton-induced hypercholesteremia (Fig. 1). Likewise, linoleyl tosylate force-fed at a level of 10 mg per day for 7 days beginning with 7-day-old animals did not overcome the hypercholesteremia characteristic of suckling rats (controls 121 ±8; linoleyl tosylate 147 ± 11; $P < 0.1$ with five animals in each group).

Linoleyl tosylate does not inhibit hepatic cholesterol biosynthesis. Dietary linoleyl tosylate did not significantly reduce the in vivo incorporation of [1-^{14}C] acetate or [5-^{3}H] mevalonate into liver cholesterol of animals maintained on either a cholesterol-free or hypercholesteremic diet (Table III). Animals fed cholesterol showed the expected lower rates of cholesterol synthesis. Linoleyl tosylate added to hypercholesteremic diet largely controlled the cholesterol levels; however, biosynthesis of sterol was still relatively slow with only a trend toward restoration being apparent. Similar re-

TABLE II

Effect of Linoleyl Tosylate on Total Body Cholesterol of Rats on a Hypercholesteremic Diet[a]

Diet	Carcass lipids			
	Total	Nonsaponifiables	Cholesterol	Nonsaponifiables not cholesterol
	g/100 g of body weight			
F-1	7.4 ± 1.1	0.9 ± 0.2	0.63 ± 0.08	0.3 ± 0.1
F-1 + 0.15% Linoleyl tosylate	4.3 ± 0.3[b]	0.7 ± 0.1	0.40 ± 0.06[b]	0.3 ± 0.1

[a]Male weanling rats were fed Diet F-1 (hypercholesteremic diet with 1% hydrogenated coconut oil) for 4 weeks. They were then divided into two groups of four rats and continued on this diet with and without supplementation (0.15% linoleyl tosylate) for 4 additional weeks. The gastrointestinal tracts were removed and cleansed with saline, and then dissolved with the carcass in aqueous 40% NaOH (0.7 ml/g tissue). The digest was extracted for total saponifiables and nonsaponifiables which were determined gravimetrically.

[b]$P < 0.05$.

sults to those of Table III were observed with liver slices prepared from animals fed in the same manner and incubated in vitro with labeled acetate and mevalonate (14).

Linoleyl tosylate increases the fecal elimination of dietary [4-^{14}C] cholesterol. The appearance of label with time in the feces of rats which had consumed a meal containing [4-^{14}C] cholesterol with and without linoleyl tosylate is shown in Table IV. There was a trend for the excretion to be greater during the first two collection periods, and the effect became statistically significant after 6 days of collection.

Linoleyl tosylate prevents the accumulation of dietary [4-^{14}C] cholesterol in blood and liver. Linoleyl tosylate decreased the rate at which label accumulated in the liver of rats fed a single meal containing [4-^{14}C] cholesterol (Fig. 2). In an experiment designed to evaluate this apparent difference statistically, considerably less radioactivity was found in both liver and blood 8 hr after feeding the tosylate-containing meal (Table V).

Oleyl p(n-decyl) benzene sulfonate prevents the accumulation of [26-^{14}C] cholesterol from the intestinal tract. Oleyl p-(n-decyl) benzene sulfonate significantly decreased the amount of radioactivity recovered in the liver 48 hr after force-feeding [26-^{14}C] cholesterol (Table VI). In contrast, more radioactivity was found in the fecal material of the treated animals. There were only slight differences in the percent recovered in the expired CO_2 and carcass.

Linoleyl tosylate increases the excretion of neutral sterols. Excretion of cholesterol and bile acids by weanling rats fed Diet I was compared with and without linoleyl tosylate over a 30 day period (Table VII). Linoleyl tosylate was found in this study to increase the amount

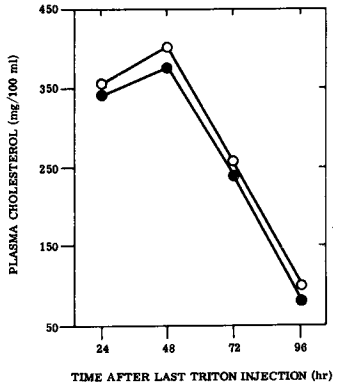

FIG. 1. Effect of linoleyl tosylate on Triton (WR-1339)-induced hypercholesteremia. Rats were maintained on Diet G-1 (cholesterol-free, 1% hydrogenated coconut oil) throughout the study with the diet of one group being supplemented with 0.15% linoleyl tosylate. The animals were starved 24 hr and then fed the diets for 3 days. Triton (0.4 cc of a 25% aqueous preparation) was then administered intraperitoneally once daily for 3 consecutive days. On the fourth day after the initial Triton injection and for 3 successive days thereafter, blood was drawn by cardiac puncture and analyzed for total plasma cholesterol. Each point represents the average for nine animals; there were no significant differences between the two groups. ○, control animal; ● linoleyl tosylate fed animals.

of fecal cholesterol excreted relative to the dietary cholesterol intake. No significant effect was found upon the amount of fecal bile acid excreted relative to dietary bile acid intake. The neutral sterol extract of the fecal material obtained from these animals was investigated in further detail. Digitonin was found to precipitate practically all (95%) of the fecal sterols which reacted with the Liebermann-Burchard reagent, suggesting that the quantity of 3α-hydroxy-sterols was negligible and not

TABLE III

Effect of Linoleyl Tosylate upon the Incorporation of [1-^{14}C] Acetate and [5-^{3}H] Mevalonate into Liver Cholesterol in vivo[a]

Diet	Liver cholesterol		Incorporation into liver total cholesterol	
	Total	Free	[^{14}C] Acetate	[^{3}H] mevalonate
	mg/g wet wt		CPM x 10^{-3}/g wet wt in 60 min	
G-2	2.2 ± 0.1	1.9 ± 0.1	8 ± 1	47 ± 4
G-2 + Linoleyl tosylate	2.4 ± 0.1	2.1 ± 0.1	10 ± 3	51 ± 4
F-2	12 ± 1[b]	2.55 ± 0.04[b]	2.7 ± 0.2[b]	23 ± 2[b]
F-2 + linoleyl tosylate	4.7 ± 0.4[bc]	2.1 ± 0.1[c]	2.8 ± 0.8[b]	35 ± 2[bc]

[a]Groups of five mature rats were fed Diet F-2 (hypercholesteremic diet with 2% hydrogenated coconut oil) or Diet G-2 (same as Diet F-2 but free of cholesterol and glycocholate) with and without 0.15% linoleyl tosylate for 12 days. An aqueous solution of [1-^{14}C] acetate (7μc) and [5-^{3}H] mevalonate (3.5 μc) was injected i.p. into each rat. Livers were removed 60 min later. Cholesterol was purified as the digitonides for both the determination of quantity and radioactivity.

[b]$P < 0.05$ with respect to animals on Diet G.

[c]$P < 0.05$ with respect to animals on Diet F.

TABLE IV

Effect of Linoleyl Tosylate upon the Fecal Elimination of a Fed Dose of [4-^{14}C]-cholesterol[a]

Days after [4-^{14}C] cholesterol consumed	Cumulative recovery of label with diet:	
	F	F + linoleyl tosylate
	Percent	
2	40 ± 4	47 ± 5
4	47 ± 3	55 ± 4
6	48 ± 3	58 ± 3[b]
8	49 ± 3	60 ± 3[b]

[a]Weanling rats were maintained on Diet F-2 (hypercholesteremic diet with 2% hydrogenated coconut oil) for 8 weeks. Following an 18 hr fast, each rat consumed 5 g of Diet F-2 containing [4-^{14}C] cholesterol (5.6×10^5 CPM) with or without 0.15% linoleyl tosylate. The respective diets were then continued ad libitum without labeled cholesterol. Feces were collected at 48 hr intervals and analyzed for total radioactivities. Results are expressed as means of the cumulative percentage of labeled cholesterol recovered for the five animals of each group.

[b]$P < 0.05$.

TABLE V

Effect of Linoleyl Tosylate upon the Amount of Radioactivity Recovered from Liver and Whole Blood 8 hr after ingestion of a Single Meal Containing [4-^{14}C] Cholesterol[a]

Diet	Percent recovered in:	
	Whole blood	Liver
F-2	0.50 ± 0.02	4.2 ± 0.2
F-2 + linoleyl tosylate	0.25 ± 0.02[b]	1.5 ± 0.1[b]

[a]The experiment was conducted as described in the legend to Figure 2.

[b]$P < 0.001$ with all values the means ± SEM with six animals in each group.

changed by the presence of linoleyl tosylate. Furthermore, no evidence was obtained for "fast-acting" sterols with the Liebermann-Burchard reagent (8) with the neutral sterol fractions, suggesting that 7-dehydrocholesterol or a similar compound is not involved in the mechanism. Likewise, no difference was found in the visible spectra of neutral sterol fractions produced by the Liebermann-Burchard reagent after standing at 25 C for 30 and 90 min, suggesting that the coprostanol to cholesterol ratio in fecal material was not altered by the presence of dietary linoleyl tosylate. This was confirmed by analysis of the extracts by thin layer chromatography, coprostanol being separated from cholesterol on Silica Gel G plates containing 3% silver nitrate with development of chloroform.

Another balance experiment was conducted to estimate the quantity of fecal cholesterol which had equilibrated with tissue cholesterol and the amount of bile acid formed by the liver. Rats in which the endogenous cholesterol pools were continuously equilibrating with [4-^{14}C] cholesterol from a pellet of gelatin were used for this experiment (Table VIII). The ratio of cholesterol specific activities suggests that the liver and blood pools were equilibrated in both control and linoleyl tosylate-fed animals. Linoleyl tosylate caused a decrease in the excretion of the combined total of "neutral sterols plus bile acids." This represents the fraction of the pools of these compounds which had equilibrated with the hepatic and blood pools of cholesterol. Separation of this fraction into saponifiable and nonsaponifiable fractions showed that animals fed linoleyl tosylate excreted less bile acid. In contrast, neutral sterol excretion was elevated significantly by

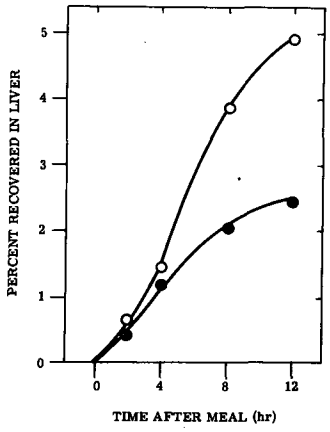

FIG. 2. Effect of linoleyl tosylate on [4-^{14}C] cholesterol accumulation in the liver. Male rats (90-105 g) were fasted for 20 hr and then each was fed 2 g of diet F-2 (1% cholesterol, 0.5% sodium glycocholate with 2% hydrogenated coconut oil) containing [4-^{14}C] cholesterol (1.54 x 10^6 CPM) with or without 0.5% linoleyl tosylate. The meal was consumed by each rat within two hr. Each point represents the average for two animals. ○, control animals; ●, linoleyl tosylate fed animals.

linoleyl tosylate.

Linoleyl tosylate stimulates the activity of intestinal mucosa cholesteryl esterase. Cholesteryl esterase, extracted from acetone powder of intestinal mucosa, was activated by linoleyl tosylate (Table IX). The greatest relative effect of linoleyl tosylate was produced when palmitic acid was the exogenous fatty acid added; however, substantial increases were also noted with unsaturated fatty acids as substrates. Linoleyl tosylate did not influence the composition of the cholesteryl esters formed.

TABLE VI

Effect of Oleyl p-(n-decyl) Benzene Sulfonate on the Distribution of Radioactivity in Tissues, Feces, and Expired CO_2 of Rats Force Fed [26-^{14}C] Cholesterol[a]

Diet	Percent of [26-^{14}C] cholesterol recovered in: Liver	Feces	Expired CO_2	Carcass	Percent absorbed[b]	Percent recovered
F-20	37 ± 2	41	5	11.2	53	94
F-20 + 0.1% ODS	8 ± 2[c]	67	7	10.6	26	93

[a]Weanling rats were maintained on Diet F-20 (hypercholesteremic diet with 10% beef tallow plus 10% safflower oil) for 7 weeks. The diet of one group of rats was then supplemented for 2 days with 0.1% oleyl p-(n-decyl) benzene sulfonate (ODS). All rats then received by stomach tube 1.1 μc [26-^{14}C] cholesterol (50 mCi/mmole) dissolved in 0.5 ml of olive oil. The animals were maintained on their respective diets for another 48 hr before sacrifice. There were six animals in the control group; five in the ODS group. Livers were analyzed separately and results are given as means ± SEM. For expired CO_2 and fecal (plus digestive tract content) radioactivity, two pools of samples were analyzed for each group. For carcass radioactivity, all the animals of each group were pooled.

[b]Percent absorbed equals the sum of the amounts recovered in the liver, expired CO_2, and carcass.

[c]$P < 0.001$.

TABLE VII

Effect of Linoleyl Tosylate upon the Quantity of Cholesterol and Bile Acid Excreted in Fecal Material Over a 30 Day Period[a]

Diet	Cholesterol found in:		Cholesterol		Cholic acid		Ratio excreted to intake:	
	Plasma	Liver	Intake	Excreted	Intake	Excreted	Cholesterol	Bile acid
	mg%	mg/g wet wt	g/rat/30 days		g/rat/30 days			
I	260 ± 20	25 ± 3	3.18 ± 0.10	2.28 ± 0.10	0.62 ± 0.02	0.69 ± 0.04	0.717 ± 0.012	1.11 ± 0.05
I + Linoleyl tosylate	54 ± 4[b]	2.9 ± 0.1[b]	2.98 ± 0.14	2.49 ± 0.13	0.58 ± 0.03	0.68 ± 0.09	0.835 ± 0.048[b]	1.18 ± 0.10

[a]Weanling rats (six per group) were fed Diet I with and without 0.15% linoleyl tosylate for 30 days. The dietary intake of control animals was restricted to be approximately that of the rats fed linoleyl tosylate.

[b]$P < 0.05$.

DISCUSSION

These studies demonstrate that arylsulfonate esters of long chain alcohols most likely exert their cholesterol lowering effects by inhibiting cholesterol absorption from the gastrointestinal tract. Another possibility, considered less likely, is that arylsulfonate esters increase the rate at which the cholesterol absorbed from the gastrointestinal tract is secreted back into the gastrointestinal tract by the route of the lymphatic system, blood system, liver, and bile. Regardless of whether these compounds act at the level of absorption in the gut, retention in the liver, or both, the evidence indicates an increase in the excretion of neutral sterols rather than bile acids as an overall effect of these hypocholesteremic agents in removing the sterol nucleus from the body.

Linoleyl tosylate is considerably more effective in the cholesterol-fed animal model system than a number of other hypocholesteremic drugs which were studied here, i.e., thyroxin (15), benzmalecene (16), ethyl α-p-chlorophenoxyisobutyrate (17), and SK & F 525A (18). Presumably these compounds work by a different mechanism than linoleyl tosylate and would show greater hypocholesteremic activity in other model systems. Previous studies from this laboratory have demonstrated that linoleyl tosylate is very effective in this model system but ineffective in the normocholesteremic rat (1,2). The hypercholesteremia of the suckling rat, as shown here, is also resistant to linoleyl tosylate.

The experiments on Triton-induced hypercholesteremia and the incorporation studies with labeled acetate and mevalonate demonstrate that linoleyl tosylate does not inhibit cholesterol biosynthesis. Since cholesterol feeding per se inhibits cholesterogenesis (19), an effect of linoleyl tosylate at this level was never considered a likely explanation for the hypocholesteremic action of linoleyl tosylate. The lack of any cholesterol-lowering response with normocholesteremic rats also supports the conclusion that linoleyl tosylate is not an inhibitor of cholesterol synthesis.

Experiments were also conducted to determine whether linoleyl tosylate caused the accumulation of some other sterol-like compound or promoted the deposition of cholesterol in extrahepatic tissues. These possibilities were also readily ruled out. Hence, linoleyl tosylate must promote the excretion of either cholesterol or its degradation products, i.e., bile acids. In support of this hypothesis, a single orally administered dose of [4-^{14}C] cholesterol was observed to appear in greater amounts in

TABLE VIII

Effect of Linoleyl Tosylate upon the Excretion of Cholesterol and Bile Salts as Determined by a [4-^{14}C] Cholesterol Balance Study[a]

Diet	Total liver cholesterol	Specific activity ratio[b]	Fecal excretion of:		
			Neutral sterols + bile salts	Neutral sterols	Bile salts
	mg/g wet wt		mg/24 hr		
H	13 ± 2	0.95 ± 0.03	33 ± 2	4.6 ± 0.5	24 ± 1
H + Linoleyl tosylate	5.5 ± 0.6[c]	1.01 ± 0.05	27 ± 2[c]	7.7 ± 0.8[c]	16.2 ± 0.6[c]

[a]Gelatin pellets containing 5 x 10^6 CPM of [4-^{14}C] cholesterol (100 mg) were inserted dorsally under the skin of 250 g rats. Animals were fed the colony diet during a 6 week equilibration period. The diet was then changed to Diet H (colony diet plus 1% cholesterol plus 0.5% sodium glycocholate) with and without 0.15% linoleyl tosylate for 14 days. On days 13 and 14, 24 hr feces collections were made. Blood and livers were taken at the end of day 14 and analyzed for cholesterol specific activity. Fecal material was dried and extracted for total radioactivity with $CHCl_3$-methanol. These extracts were hydrolyzed and separated to saponifiable and nonsaponifiable fractions which were assayed for total radioactivity. Calculations were then based upon the equations given in the text.

[b]Ratio of the specific activity of total liver cholesterol to total blood cholesterol.

[c]$P < 0.05$, values are the means ± SEM with five to six animals in each group.

TABLE IX

Effect of Linoleyl Tosylate upon Cholesterol-4-C^{14} Esterification in the Absence and Presence of Exogenous Fatty Acids[a]

Exogenous fatty acid added	Linoleyl tosylate added	% Cholesterol-4-C^{14} esterified	% Cholesterol-4-C^{14} found esterified to:		
			Palmitate	Oleate	Linoleate
None	-	0.9	40.9	30.6	28.5
	+	5.0	43.6	29.5	26.9
Palmitic acid	-	0.8	66.2	16.7	17.1
	+	7.0	68.1	13.1	18.8
Oleic acid	-	9.8	20.9	66.6	12.5
	+	13.3	20.6	60.2	19.2
Linoleic acid	-	5.2	15.7	11.8	72.5
	+	11.2	12.8	10.9	76.4
Linoleic + palmitic acid	-	5.6	36.0	8.3	55.7
	+	11.4	44.3	6.9	48.8

[a]Cholesterol-4-C^{14} (5 μmoles) was incubated for 3 hr with an extract of an acetone powder of small intestine from rats fed colony diet. Fatty acids were added in amounts of 3.5 μmoles; linoleyl tosylate, 10 μmoles. Each value is the average of duplicate samples.

the feces of linoleyl tosylate fed rats. Evidence was also obtained that the action of linoleyl tosylate is directed towards increased excretion of neutral sterol rather than bile acids. This conclusion is based on balance studies with rats fed cholesterol and glycocholate in which linoleyl tosylate was found to significantly increase the ratio of excreted cholesterol to consumed cholesterol but to be without effect upon the same ratio for cholic acid. Likewise, in balance studies in which the hepatic and serum pools of cholesterol were continuously equilibrating via an implanted pellet or labeled cholesterol, evidence was obtained for increased excretion of the neutral sterols rather than bile acids. In addition, the experiment carried out with [26-^{14}C] cholesterol supports this conclusion. Oleyl p-(n-decyl) benzene sulfonate decreased the appearance of radioactivity in the liver of animals force-fed [26-^{14}C] cholesterol but increased the appearance of radioactivity in fecal material. These results do not suggest that there is increased bile acid formation. Side chain cleavage during bile acid formation would liberate the radioactivity of the [26-^{14}C] cholesterol. An enhanced bile acid formation, caused by the arylsulfonate, would be expected to decrease the amount of fecal radioactivity. Since greater fecal radioactivity was observed in the treated animals, the results are consistent

with an inhibition of absorption.

An important observation of this study was that linoleyl tosylate greatly decreased the rate at which [4-^{14}C] cholesterol from a single meal accumulated in the liver and blood. This implies that linoleyl tosylate inhibits cholesterol absorption from the gastrointestinal tract. Cholesterol is absorbed in the free form from the intestinal lumen, esterified in part in the intestinal mucosal cells, transported as the ester as part of the chylomicrons in the lymph to the blood stream, and eventually taken up primarily as the ester by the liver (20). Cholesteryl esters can be stored in the liver, released in the blood associated with lipoproteins, or hydrolyzed to free cholesterol by cholesteryl ester hydrolase. Unesterified cholesterol can be oxidized to bile acids, re-esterified by cholesteryl ester synthetase, released into the blood associated with lipoproteins, or shunted into the bile to return to the intestinal tract. Since linoleyl tosylate inhibits the accumulation of cholesterol in serum as well as the liver, there is no evidence that the tosylate-treated animals release cholesterol more rapidly into the blood. Although an inhibition of the absorption of cholesterol by linoleyl tosylate is favored by the results of these studies, the possibility that linoleyl tosylate might increase the rate at which absorbed cholesterol is cleared by the liver and recirculated back to the intestinal tract by way of the bile is not excluded.

It has been reported that certain other synthetic derivatives of linoleic acid, notably N-cyclohexyl linoleamide, N-(α-methylbenzyl) linoleamide (AC–223), and (-)N-[α-phenyl-β-(p-toyl)ethyl] linoleamide (AC-485) also possess hypocholesterolemic properties (21-26). These compounds decrease plasma cholesterol levels of cholesterol-fed rats or rabbits and inhibit atheromatous changes of rabbit aorta. These drugs have also been reported to depress exogenous cholesterol appearance in the thoracic chyle, in peripheral blood, and in the liver subsequent to oral administration of labeled cholesterol (27,28). In addition, N-cyclohexyl linoleamide does not inhibit cholesterol synthesis from labeled acetate or mevalonate by rat liver slices (29,30). Also, as reported here for linoleyl tosylate, (-)N-[α-phenyl-β-(p-toyl)ethyl] linoleamide has been shown to increase the esterification of cholesterol by mucosal cholesterol esterase (31). Furthermore, it has been suggested that inhibition of cholesterol absorption by the linoleamides is directly related to their effects on the mucosal cholesteryl esterase (31). The linoleamides and arylsulfonate esters appear to have similar effects on cholesterol metabolism and may, therefore, have the same mechanism of action. Obviously, additional experiments are necessary in order to clearly define the mechanism(s) of action of these compounds.

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The Occurrence and Distribution of Furan Fatty Acids in Spawning Male Freshwater Fish

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ABSTRACT

Furan fatty acids (F acids) have been found in the livers and/or testes of 20 species, representing 9 families, of male freshwater fish. In 9 species they are major components of the lipids while in the remaining 11 species they occur to a much lesser extent. The F acids in some species reach a maximum concentration in the testes lipids, and minimum liver lipid concentration, at spawning. In all species in the testes, the F acids are confined almost exclusively to the triglyceride fraction while, in the liver lipids, they are found, in order of decreasing concentration, in the cholesteryl esters, the triglycerides, and the phospholipids. In the lipids of many individuals F_6, 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid, is the major fatty acid present. It is presumed that these acids perform some as yet unidentified metabolic function. Isolation technology and identification of F acids by a specific thin layer chromatographic spray reagent are discussed.

INTRODUCTION

We have recently reported on the occurrence and structure of a novel series of fatty acids in the organs of certain freshwater fish (Glass et al., 1,2). There are at least 8 members of this series which are characterized by the presence of either a tri- or a tetrasubstituted furan ring. The principal member, F_6, is 12,15-epoxy-13,14-dimethyleicosa-12,14-dienoic acid shown below. The furan fatty acids, F acids, differ from one another in the presence or absence of a methyl group on the ring at carbon 4 and in the length of the chains on ring carbons 2 and 5 (Table I). Apparently the only known naturally occurring similar compound is the disubstituted furan acid 9,12-epoxy-octodeca-9,11-dienoic acid found by Morris et al. (3) in the seed oil of *Exocarpus cupressiformis*. Gunstone et al. (4) and Scrimgeour (5) have since reported finding the F acids in liver oils from a number of marine fish.

In our earlier paper (1), we reported the presence of the F acids in 5 species of fish. The present communication extends the list to 20 species representing 9 families.

MATERIALS AND METHODS

Fish and Fish Lipids

All fish, except the trout, were obtained by angling or netting from lakes and rivers within a 75 mile radius of St. Paul, MN. The trout had been raised from fry in an outdoor pond and were about 1-year-old at the start of our observation in October 1974. They had been fed largely on fathead minnows occasionally supplemented with ground carp. The fish were transported live or packed in ice to the laboratory and the organs worked up, in most cases, within 2-3 hr of death. The organs were excised and homogenized in a blender with chloroform:methanol (2:1, v/v) and the procedure continued according to the aqueous wash method of Folch et al. (6). The recovered lipids were dissolved in methanol:benzene (1:1 v/v) to make either a 5 or 10% solution and stored at -10 C under nitrogen. Fractionation of the lipids into the several main classes by preparative thin layer chromatography (TLC), the preparation of methyl esters by alkaline methanolysis, their analyses by gas liquid chromatography (GLC), and the identification of fatty acid methyl esters by mass spectrometry using a LKB-9000 (GC-MS) instrument were as described earlier (1).

Argentation TLC

As earlier reported by us (2) and subsequently verified by Gunstone et al. (4), the F acids are not retarded appreciably during argentation chromatography. Their methyl esters migrate with those of the saturates and monoenes. This permits a ready separation of these esters from those of the polyunsaturated fatty acids, (PUFA) on preparative plates consisting of Silica Gel H containing 5% $AgNO_3$ (w/w). Following development in hexane:diethyl ether (75:25, v/v), the material in the leading band is eluted with diethyl ether, and the partially

[1]Scientific Journal Series 9910, Agricultural Experiment Station, University of Minnesota, St. Paul, MN 55108.

purified F acids so obtained can then be further purified by precipitating the saturates and monoenes with urea-methanol as previously described (1).

Rapid Column Argentation

More routinely useful is the following procedure developed because of difficulty frequently encountered in identifying and quantitating the F acid methyl esters which elute with those of the PUFA during GLC. Activated Silica Gel H containing 5% $AgNO_3$ (w/w) is dusted into a 1 cc tuberculin syringe filling it to the 0.6 cc mark, about 0.5 g. With the syringe clamped in a vertical position and fitted with a 22 g., 1" needle, 50 µl of a 5-10% solution of methyl esters, dissolved in either hexane or methanol:benzene (1:1, v/v) are placed on the column. As soon as the liquid has gone into the column, the space over it is filled with hexane: diethyl ether (75:25, v/v). The first 0.2 ml, which takes 2-3 min by gravity flow, are collected for analysis by GLC. This contains virtually all of the non-PUFA including the F acid esters. The material remaining on the column may be eluted with methanol or the column simply discarded. The entire procedure takes less than 10 min.

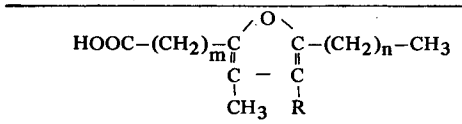
Caution is indicated in the evaluation of lipid samples following argentation chromatography. Chen et al. (7) reported the formation of 1,2-epoxides from unsaturated fatty acid methyl esters when diethyl ether with a peroxide content of 14 meq/kg was used in the solvent mixture. Such compounds could conceivably be misidentified as furan fatty acids. More pertinent was the report by Crundewell and Cripps (8) that hydroxy-*cis*-enynoic acids are readily converted to furan type acids by treatment with alkali or by argentation chromatography. These authors suggested that the furan acid found by Morris et al. (3) may have been an artifact of the isolation procedure-a possibility, in fact, which had been raised by Morris et al. The fish furan acids reported here are present prior to argentation chromatography and are found when procedures not involving diethyl ether or alkali are used.

Color Detection of TLC

A spray reagent for the detection of the F acids in lipid mixtures has been suggested (H. Schlenk, private communication). The developed plate is sprayed with a 2% (w/v) mixture of tetracyanoethylene in acetone. The F acids appear as blue spots on a pale yellow background. The reagent is nondestructive and the material may be subsequently analyzed by GLC in the usual mammer. Used as described, the reagent is sensitive above about 5% of F acids in the mixture of lipids.

TABLE I

The Structure of the Furan (F) Acids



Compound	m	n	R
F_1	8	2	CH_3
F_2	8	4	H
F_3	8	4	CH_3
F_4	10	2	CH_3
F_5	10	4	H
F_6	10	4	CH_3
F_7	12	4	H
F_8	12	4	CH_3

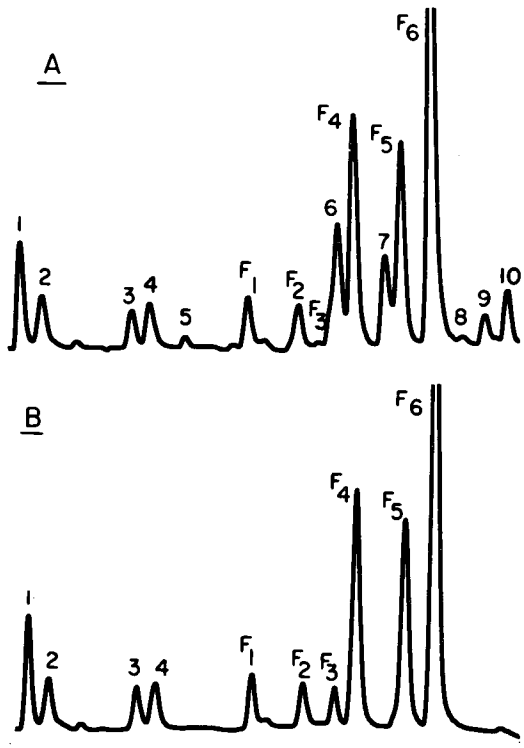


FIG. 1. A. Chromatogram of northern pike testes lipid fatty acid methyl esters, B. esters following chromatography on $AgNO_3$-silica gel. 1. 16:0; 2. 16:1; 3. 18:0; 4. 18:1; 5. 18:2; 6. 20:4; 7. 20:5; 8. 22:5ω6; 9. 22:5ω3; 10. 22:6.

RESULTS

Figure 1 shows chromatograms of methyl esters from northern pike testes lipids before

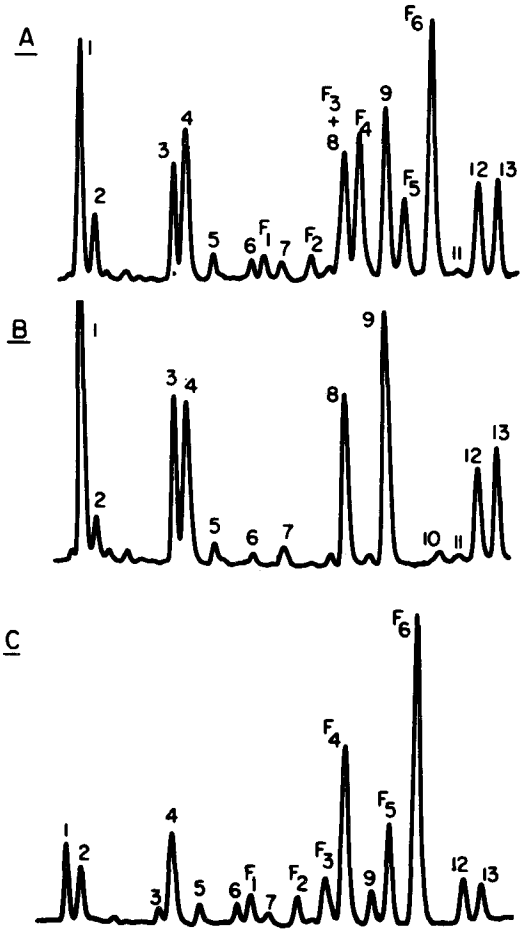


FIG. 2. Chromatograms of carp testes fatty acid methyl esters. A total fatty acids, B. phospholipid fatty acids, C. triglyceride fatty acids. 1. 16:0; 2. 16:1; 3. 18:0; 4. 18:1; 5. 18:2; 6. 18:3; 7. 20:1ω9; 8. 20:4ω6; 9. 20:5ω3; 10. 22:4ω?; 11. 22:5ω6; 12. 22:5ω3; 13. 22:6ω3.

(Fig. 1A) and after (Fig. 1B) the $AgNO_3$-silica gel column chromatographic procedure described. The F acid esters, which constituted about 57% of the total, have been isolated from the PUFA whose presence prevented an accurate assessment of the relative amounts of F acids. In particular, F_3 which appears only as an asymmetry on the leading edge of 20:4ω6 in 1A is completely isolated in Figure 1B. Such a preparation, made on a larger scale, is suitable for further purification by preparative GLC.

Figure 2 shows the pronounced difference in fatty acid composition between the several lipid classes encountered in the presence of F acids. Figure 2A shows a chromatogram of the total fatty acid methyl esters obtained from carp testes lipids. The F acid content was about 38%. The phospholipids (PL), Figure 2B, contained essentially no F acids while the triglycerides (TG), Figure 2C, contained 65.4% F acids. We frequently encounter samples in which the triglycerides consist of in excess of 95% F acids.

The F acids have been found in the livers of males of 10 of the 11 species listed in Table II and in the livers, testes, or both in the 9 species listed in Table III. Table II lists those species in which the F acids are presently considered minor components or which have not yet been extensively studied. Each individual listed had the highest F acid content observed for that species. In all cases, as shown, the F acids were concentrated in the cholesteryl ester (CE) fraction with much smaller amounts in the TG and essentially none in the PL (not shown). The mean F acid content of all samples listed in Table II was 3.6% total, 44.4% CE, and 1.5% TG. We have observed no case where the liver TG F acid content was higher than that of the CE. The minnow was an extract of 50 whole fish. The livers of this species have not yet been examined. Only the testes lipids of the black crappie have thus far been observed to contain F acids. Yet to be examined are testes lipids of the fathead minnow and the catfish. The fish listed in Table II will be further examined as they become available.

The 9 species in Table III will be discussed individually. These are fish which have either been examined more thoroughly than those listed in Table II or in which elevated levels of the F acids have been found in the few fish examined (rock bass, carpsucker, and bullhead).

Brook Trout

This fish spawns in the fall (Oct.-Nov.). Some time prior to that (June-Aug.), the testes, in an atrophied condition since last spawning, begin to enlarge, and the males become brightly colored and readily distinguishable from the females. Concurrent with these changes, and presumably under hormonal influence, the livers, which have contained 15-20% fat whose fatty acids include 10-15% F acids, decrease markedly in both. At spawning, the livers contain 4-6% fat and 0-5% F acids. The enlarging testes accumulate F acids as shown in Table III. The range in F acid content observed in the 12 individuals averaged in Table III was 15-35% of the total testes fatty acids. Since the decrease in liver F acids coincides with their accumulation in the testes, it is reasonable to conclude that they have been transported from the former to the latter. In support of this, blood lipids of a single trout taken in August, at which time the translocation was presumably occurring, was

TABLE II

Furan Acids as Minor Components in Individual Male Freshwater Fish Liver Lipids[a]

Fish and common name (9)	Month	Liver furan acids, % of total fatty acids[b] Total	CE[c]	TG[c]
1. *Salmo gairdneri* Rainbow trout	Aug	6.7	59.7	0.7
2. *Pimephales promelas* Fathead minnow	Nov.	1.1	11.8	0.0
3. *Osmerus mordax* Smelt	May	3.1	34.9	0.0
4. *Micropterus dolomieui* Smallmouth bass	Sept	0.8	37.6	0.0
5. *Stizostedion vitreum* Walleye	June	5.5	83.2	1.2
6. *Perca flavescens* Yellow perch	June	T[d]	17.4	0.0
7. *Ictalurus punctatus* Channel catfish	Mar	1.7	39.2	0.0
8. *Micropterus solmoides* Largemouth bass	Sept	4.7	48.6	2.5
9. *Pomoxis nigromaculatus* Black crappie	June	3.7	10.1	2.4
10. *Catostomus commersoni* Common white sucker	Mar	6.9	79.2	5.5
11. *Moxostoma macrolepidotum* Shorthead redhorse	Sept	5.0	67.2	3.9

[a]Minnow lipids obtained from 50 ground whole fish.

[b]Calculated as methyl esters, by weight.

[c]CE = cholesteryl esters, TG = triglycerides.

[d]T = trace, <0.3%.

TABLE III

Furan Acids as Major Components in Freshwater Fish Lipids

Fish and common name (9)	Month	No. fish	Furan acids, % of total fatty acids Liver	Testes
1. *Salvelinus fontinalis* Brook trout	Oct	12	T[a]	22.5 ± 9.1
2. *Esox lucius* Northern pike	April	18	2.6 ± 2.9	± 43.4 ± 10.8
3. *Cyprinus carpio* Carp	May	22	0.6 ± 1.5	24.3 ± 11.3
4. *Ictiobus cyprinellus* Bigmouth buffalo	March	21	26.0 ± 7.2	T[a]
5. *Lepomis macrochirus* Bluegill	July	74	15.2 ± 11.6	T[a]
6. *Ictalurus melas* No. black bullhead	July	6	13.0 ± 9.4	N.A.[b]
7. *Carpiodes velifer* Carpsucker	April	6	8.2 ± 4.5	T[a]
8. *Amia calva* Bowfin (Dogfish)	March	7	2.7 ± 0.5	9.9 ± 1.4
9. *Ambloplites rupestris* Northern rockbass	Aug	1	22.2	N.A.[b]

[a]T = Trace, <0.3%.

[b]N.A. = not available.

found to contain 8.5% of its total fatty acids as F acids. These were entirely in the CE fraction where they comprised 28.0% of the fatty acids.

Fractionation of the lipids pooled, respectively, from the testes and livers of 6 fish into classes (No. 1, Table IV), and analyses of the several classes showed that the testes F acids were largely in the TG whose fatty acids were

TABLE IV

The Furan (F) Acid Distribution in Spawning Freshwater Fish[a]

					F acids, % of total fatty acids in lipid class				Individual F acids as percent of total F acids					
		Month	Lipid %	mg/g[b]	OR	CE	TG	PL	F_1	F_2	F_3	F_4	F_5	F_6
1. Brook trout[c]														
	testes	Oct	3.6	10.0	34.7	4.4	57.5	2.5	–	–	–	2.3	19.9	77.8
	liver		4.8	0.3	0.7	10.3	0.0	0.0	–	–	–	10.7	24.3	65.0
2. Northern pike														
	testes	Mar	3.6	14.4	50.0	N.P.	84.1	0.0	4.6	6.6	T	21.2	10.8	56.8
	liver		4.4	1.7	4.8	84.1	2.0	0.0	–	–	–	13.3	–	36.7
3. Carp														
	testes	May	6.4	19.9	46.0	N.P.	61.9	0.0	4.1	4.6	T	24.1	18.5	48.7
	liver		3.8	3.2	0.8	50.3	0.1	0.0	–	–	–	27.8	20.0	52.0
4. Bigmouth Buffalo														
	testes	Feb	2.7	0.6	2.8	12.3	7.4	0.0	10.7	32.1	T	T	25.0	32.1
	liver		6.8	19.9	36.6	96.0	39.3	0.0	1.6	12.6	T	15.0	12.0	58.7
5. Bluegill														
	testes	July	7.7	2.2	4.0	41.4	3.5	6.3	–	12.5	–	20.0	–	67.5
	liver		9.6	18.0	23.4	91.9	4.2	3.7	T	4.7	T	35.9	2.1	57.3
6. Bullhead														
	testes	July			none examined									
	liver		4.5	10.9	30.4	95.8	14.1	0.0	–	2.3	–	27.6	5.3	64.8
7. Carpsucker														
	testes	Apr			T									
	liver		6.5	7.6	14.6	86.7	12.0	1.9	T	17.8	T	17.8	24.0	40.0
8. Dogfish														
	testes	Mar	2.1	1.7	10.2	17.6	37.0	2.3	2.9	31.4	T	T	20.6	45.0
	liver				T									
9. Rockbass[d]														
	testes	Sept			0.0									
	liver		3.6	6.4	22.2	86.3	19.0	11.9	T	6.8	T	16.7	10.4	66.2

[a]OR = total F acids, CE = cholesteryl ester F acids, TG = triglyceride F acids, PL = phospholipid F acids, N.P. = not present .

[b]Calculated on assumption that the lipids were 80% fatty acids.

[c]Brook trout a pool of lipids from six fish, all others lipids from individual fish.

[d]Probably post-spawning.

57.5% F acids. The pooled liver lipids from the same fish contained less than 1% of F acids which were entirely in the CE fraction. Only $F_{4,5,6}$ were present in either tissue with F_6 predominating to the extent of 77.8% in the testes lipids and 65.0% in the liver lipids.

Since the total amount of the F acids present in a tissue is a function not only of its percentage of the total fatty acids but also of the lipid content, an estimate of this was made for which it was assumed that the lipids were 80% fatty acids. On this basis, as shown in Table IV, trout testes contain, at spawning, 10.0 mg/g F acids and the livers 0.3 mg/g. The latter is much reduced from the ca. 10-12 mg/g liver observed in trout in July and August.

Northern Pike

This fish spawns in the early spring (March-April). It is a carnivore feeding on other fish. The pike shows the same fluctuations in F acid content and distribution observed in the brook trout. At spawning the liver lipids, which in October and November contain as much as 50-60% of their total fatty acids as F acids, contain less than 3% F acids as shown in Table III. The testes, which have increased 10- to 20-fold in size over their condition following the last spawning, contained, for 18 fish, an average of 43.4% F acids. The distribution of F acids in the several lipid classes of a single fish is shown in Table IV. We do not find appreciable amounts of CE in the lipids of northern pike testes and the F acids are largely confined to the TG. In this individual, the F acids comprised 50.0% of the total fatty acids and 84.1% of the TG fatty acids. All 6 of the series were present with F_6 (56.8%) and F_4 (21.2%) predominating. The testes contained 14.4 mg/g of F acids.

The liver lipids of the same individual contained only 4.8% F acids which were largely in the CE and consisted entirely of F_6 and F_4. F_7 and F_8 reported earlier (1) cannot be seen without removal of the C22 unsaturates and this is not routinely done. Like the brook trout, a high liver F acid content is usually accompanied, in the northern pike, by an elevated lipid content. Thus, for the spawning fish listed in Table III, the mean liver lipid content was 4.4% and the F acid content was 0.7% or ca. 0.25 mg/g liver. The mean liver lipid content of 11 fish caught in Oct.-Nov. was 13.7%, the mean F acid content was 41.7%, and the calculated liver content of F acids was 45.7 mg/g or 167-fold greater than at spawning. As with the trout, we find F acids in the blood of northern pike. In a single individual caught in November, the F acids comprised 13.0% of the blood serum fatty acids. The CE fraction was 29.1%, TG 13.0%, and the PL contained none.

Carp

The carp is considered an omnivore but from our observations it apparently subsists to a large extent on plant material. Unlike the trout and the pike, carp testes do not decrease noticeably in size following spawning in spring. Apparently they continue to spawn at least throughout the summer. The females contain roe the year around. Perhaps as a result of this the carp does not show the fluctuations in F acid content seen in the fish described thus far. Thus, while the mean F acid content of the testes lipids of 143 fish examined during a one-year period was 21.4 ± 8.5%, those of 22 fish caught in May (No. 3, Table III) were 24.3 ± 11.3% or essentially the same. Since the testes F acid content remains virtually unchanged during the year, one might suspect that the liver lipids F acid content are similarly rather constant but low and this is the case. Although several have been observed with liver lipid F acid contents of 8-10%, the mean for all samples obtained throughout the year was 1.1 ± 1.5%.

The distribution of F acids in a typical carp, No. 3, Table IV, is almost identical to that seen in northern pike at spawning. In the testes lipids, they are confined to the TG, there being virtually no CE present, and all 6 are present with F_6 and F_4 predominating to the extent of 72.8% of the total. This individual contained almost 20 mg F acids/g testes. In the liver lipids, only $F_{4,5, \text{ and } 6}$ were present and these almost entirely in the CE whose fatty acids consisted of about 50% F acids.

Bigmough Buffalo

The buffalo eats aquatic insect larvae, small mollusks, and vegetation. It spawns in late April and May. This fish differs from those already described in that, while the liver lipids contain large amounts of F acids (as much as 40% of the total fatty acids), the testes lipids contain very little even at spawning. The liver lipids of 130 buffalo examined throughout the year contained 17.3 ± 6.4% F acids while the highest testes lipid F acid content was less than 3%. The liver lipids of 21 fish caught just prior to spawning, Table III, were only slightly higher in F acid content than the yearly mean. The testes lipids of these fish contained essentially no F acids.

The distribution of the F acids in the liver and testes of one individual is shown in Table IV. In the liver, which contained 19.9 mg F acids/g liver, the F acids were in both the TG and the CE in which last they comprised 96%

of the total fatty acids. They were absent from the PL. All 6 of the series were represented with F_6 and F_4 predominating as with the fish already discussed. F_2, however, was present in amounts larger than found in most fish. This was true also of the testes lipids where the amount of F_2 equalled that of F_6.

Bluegill

The mean F acid content of the liver lipids of 74 bluegills caught in July was 15.2% (range 3.4-48.8%). A high F acid content is frequently accompanied by a high fat content as has also been observed with the northern pike and brook trout. When the data were divided into 2 groups of equal size on the basis of fat content, this becomes apparent as shown below.

No. samples	Fat content %	F acid content %	mg F/g liver
25	3.3 ± 1.0	10.3 ± 10.0	2.7
25	8.1 ± 2.4	23.9 ± 11.5	15.5

Fractionation studies, No. 5, Table IV, gave typical results–i.e., the F acids were concentrated in the CE fraction (91.1%) and very little were found in the TG and PL fractions. F_4 and F_6 predominated to the extent of 93.2% of the total.

Northern Black Bullhead

This species spawns in May-June. It is considered omnivorous but apparently fish are not important in their diets. The bullhead is unusual in that, apparently, testicular development is rapid, with regression quickly following spawning. In any event, we have not yet been able to examine the testes of this species. Individuals of unknown sex from June-December have been found to contain F acids in their liver lipids in amounts ranging from 10-30%. The liver lipids of 6 individuals caught in July, No. 6, Table III, averaged 13.0% F acids. Females do not contain F acids to any appreciable extent. Fractionation of the liver lipids of one individual, No. 6, Table IV, showed the F acid distribution to be similar to that of other species with F_6 predominant and the CE fraction particularly high (95.8% F acids).

Carpsucker

This species spawns in April-May. It is a bottom feeder with indiscriminant feeding habits eating both plant and animal matter. The testes lipids of 6 spawning fish, No. 7, Table III, contained only traces (<0.5%) of F acids while the liver lipids of the same fish averaged 8.2% (range 1.4-14.6%). The F acids were concentrated in the CE (Table I) and to a much lesser extent in the TG. F_2 and F_5 were present in amounts larger than that usually seen.

Bowfin

The only surviving species of its family, this fish spawns in May-June. It is a carnivore feeding exclusively on other fish. We have not examined the bowfin as extensively as most of the others listed in Table III. The testes lipids of 7 spawning males contained an average of 9.9% F acids whereas the liver lipids of the same fish contained only traces of these compounds. The F acids were largely in the TG, Table IV, with lesser amounts in both the CE and PL. Unusually large amounts of F_2 were present (31.4%) and only traces of F_3 and F_4.

Rockbass

This species spawns in May-June and feeds on small crustaceans and fish. Only two rockbass have been examined and are included here because of the high (22.2 and 24.0%) F acid content of their liver lipids. The testes lipids had essentially none but the two were caught, after spawning, in August. The F acids were present in quantity in all three fractions, Table IV, but as in most species, the CE content was highest (86.3%).

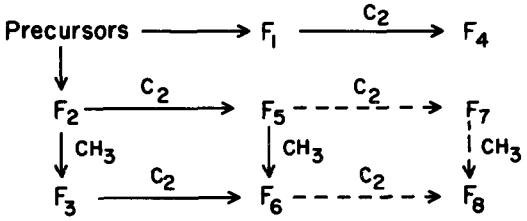
DISCUSSION

The source and function of the F acids remain unknown. The data presented, however, permit some speculations to be made. It is difficult to conceive that a group of compounds present in such large quantities and confined, essentially, to only 2 organs, liver and testes, is there without function or purpose. An external source of these compounds cannot be discounted and, indeed, when fed to fish the F acids accumulate in the liver (R.L. Glass, unpublished observations). It would be difficult to rationalize a single external source of the F acids, however, since the several species in which they are found in the highest concentration have very different dietary habits, i.e., the carp and the northern pike. Furthermore, the maximum liver concentration in some species with rather similar dietary habits is reached at different times of the year, i.e., the northern pike in November, the bluegill in July, and the brook trout in September.

In some species, most notably the brook trout and the northern pike, following spawning, the F acids begin to accumulate in the livers which become fatty. Prior to spawning, the liver content of F acids decreases markedly and the testes enlarge and accumulate them. This is suggestive of liver synthesis and trans-

port to the testes. The presence of F acids in the blood of these species during the transition period lends credence to this suggestion. Although it is tempting to suggest that the F acids perform some function during the fertilization process, such would be pure speculation.

The structures of the F acids, Table I, immediately suggest several routes by which they may be formed, one from the other as shown below. It is considered that there are 2 families, an $\omega 4$ family which includes only F_1 and F_4, and an $\omega 6$ family which includes all of the rest. The scheme presented below suggests that F_1 and F_2 are formed from precursors which are, however, probably not the same since it would appear likely, Table I, that F_1 would arise from cyclization of a 16-carbon acid and F_2 from an 18-carbon acid.



Interrelations between the Furan Acids

F_1 by the addition of acetate could be converted to F_4. Both of these acids contain 2 ring methyl groups. We have found no other members of this family and F_4, which is always present in amounts exceeding F_1, appears to be a terminal compound. F_6, which is always the major member of the $\omega 6$ family, differs from F_4 in chain length and the position of the furan ring. As shown, it could arise either from the methylation of F_5 or the elongation of F_3 each of which, in turn, could be formed from F_2. F_7 and F_8 are normally not seen without special search. Presumably they are formed only in very small amounts, as shown, or are rapidly utilized once formed. The consistent observation that F_6 comprises from 40-60% of the total F acids would tend to eliminate the last possibility. It is, of course, conceivable that F_6 and F_4 are the first formed and that the others arise from them by the reverse of the reactions shown with the exception of F_7 and F_8.

The peculiarities of the distribution of the F acids deserve mention. In testes of all species, where found, the F acids are almost exclusively esterified to glycerol as triglycerides. Essentially none are found in the phospholipid and only very small amounts of cholesteryl esters are ever present in testes lipids. These usually contain only small amounts of F acids. In liver lipids, the distribution of the F acids between

TABLE V

The Distribution of Furan Acids at Different Levels of Total Furan Acid Content in Male Northern Pike Livers

	Furan acids, %[a]		
Total	CD	TG	PL
2.4	81.5	0.0	0.0
5.8	82.5	0.0	0.0
8.8	82.8	5.2	T[b]
12.6	90.2	9.3	1.1
18.2	91.8	14.4	0.0
23.6	91.0	22.6	1.9
29.0	91.7	31.5	2.7
33.0	83.2	38.4	6.3
52.9	90.5	58.3	9.2
59.7	90.2	74.8	14.7

[a]Calculated as percent of the total methyl esters present in each fraction: CE = cholesteryl esters, TG = triglycerides, PL = phospholipids.

[b]T = Trace.

the lipid classes depends upon the total F acid content as shown in Table V. Here are listed the contribution of F acids to the total fatty acids in several liver lipids obtained from northern pike and in the three lipid classes obtained from each. The samples were selected to have increasing F acid contents. The CE fractions contain in excess of 80% F acids at all levels of total F acid content. Below 10% total F acid content, the TG fractions contain little F acids, but their content of F acids rises sharply at total F acid contents above 10%. The PL fraction similarly contains little of the F acids until the total content exceeds 30%. This last is in contrast to northern pike testes lipids where even when the total F acid content is as high as 60% virtually none is in the PL fraction. The liver phospholipid F acids were about equally distributed between phosphatidyl choline and phosphatidyl ethanolamine.

We have repeatedly observed an unequal distribution of the several F acids in liver CE and TG of all species in which the F acids are found. The CE F acids tend to be restricted to F_4 and F_6 while the TG fraction will have all of them. Thus, in a typical example, the CE fraction of a northern pike liver contained 82.9% of its fatty acids as F acids. Of these, 87.1% were $F_4 + F_6$ and 12.9% the other F acids. In the TG fraction, which contained 56.3% of its fatty acids as F acids, 57.8% were $F_4 + F_6$ while 42.2% were the others in the series ($F_{1,2,3,5}$). These observations imply a considerable specificity in esterification of F acids or in their production at specific sites.

We have occasionally observed the F acids in female livers. In particular, northern pike

females in Sept.-Nov. contain rather large amounts of them. These observations will be detailed in a future publication.

ACKNOWLEDGMENTS

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Autoxidation of Methyl Linolenate: Analysis of Methyl Hydroxylinolenate Isomers by High Performance Liquid Chromatography

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ABSTRACT

The mixture of methyl hydroxylinolenates obtained by reduction of the hydroperoxide isomers formed by autoxidation of methyl linolenate was resolved by high performance liquid chromatography into eight major components. These are positional isomers with the hydroxyl group at positions 9, 12, 13, and 16. Two geometrical isomers of each positional isomer are present; these differ in the configuration of the conjugated double bonds (*cis-trans* and *trans-trans*). Autoxidation of methyl linolenate is regioselective and favors the formation of positional isomers 9 and 16.

INTRODUCTION

The analysis by high performance liquid chromatography (HPLC) of the mixture of isomers of hydroperoxides formed by autoxidation of methyl linoleate and the corresponding mixture of hydroxy compounds (methyl hydroxylinoleates) derived (by $NaBH_4$ reduction) from them has been reported recently (1). Using adsorption chromatography on 5 μm silica particles, the hydroperoxides and hydroxy compounds were separated into individual components, thereby permitting a rapid and quantitative analysis of the composition of positional and geometrical isomers present in the mixture. Only four major isomers were produced by autoxidation of methyl linoleate and these were the 9- and 13-diene hydroperoxides having the double bonds in either *trans-trans* or *cis-trans* (*trans* double bond adjacent to the hydroperoxide group) configuration. Oxygenation therefore occurs at the ends of the 1,4-diene moiety of the linoleate molecule.

Autoxidation of methyl linolenate is more complex since two separate 1,4-diene systems are present. In a previous investigation, Frankel et al. (2), using countercurrent extraction and partition chromatography to isolate hydroperoxides, showed by chemical degradation that the major components in the mixture of hydroperoxides obtained from autoxidation of methyl linolenate were isomers having the hydroperoxide group in the 9, 12, 13, and 16 positions. Attack of oxygen occurs, therefore, at the ends of the two 1,4-diene systems, viz., between carbon atoms 9 & 13 and 12 & 16. In either case, the third double bond remains unaffected and autoxidation of linolenate is expected to be twice as complex as that of linoleate in terms of the number of isomers formed. The ability to separate the individual isomers of hydroperoxy and hydroxy dienes in the methyl linoleate series using HPLC suggested that this technique might have the resolution required for the analysis, as distinct components, of the corresponding mixtures obtained from linolenate autoxidation. This possibility has been investigated and the results are now reported.

MATERIALS AND METHODS

Methyl linolenate (98%) was obtained from Lipid Supplies (St. Andrews, UK) and Hiflosil from Applied Sciences Labs. Inc. (State College, PA).

Ultraviolet (UV) spectra were recorded in ethanol solution on a Pye-Unicam SP800 and infrared (IR) spectra (in CCl_4 solution) on a Pye-Unicam SP200 G spectrophotometer. Mass spectra were obtained from a GEC/AEI MS 902 mass spectrometer.

High Performance Liquid Chromatography (HPLC)

HPLC was carried out on 5 μm silica particles (Partisil-5) as previously described (1). For the isolation of quantities (1-3 mg) of individual components, ca. 1 mg of the mixture of methyl hydroxylinolenates was repeatedly injected. Chromatography of larger quantities resulted in substantial loss of resolution.

Preparation of Methyl Linolenate Hydroperoxide and Methyl Hydroxylinolenate Isomers

Methyl linolenate was autoxidized (40 C) in a Warburg apparatus until oxygen uptake reached ca. 5 mole % (20-40 hr). Iodometric titration (3) of the product yielded a peroxide to oxygen-uptake ratio of 0.82. The mixture of hydroperoxides was isolated by partition

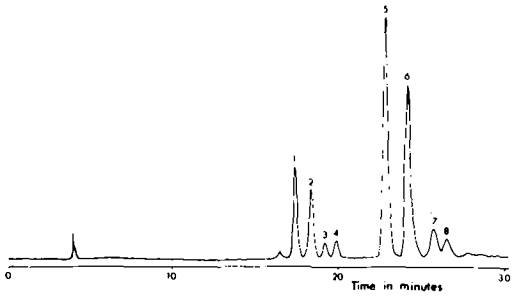


FIG. 1. High performance liquid chromatography of isomers of methyl hydroxylinolenate. Column packing: Partisil-5; eluting solvent: 0.75% ethanol in hexane; rate of solvent flow: 4 ml/min. Structures of isomers: 1, 13-*cis-trans;* 2, 12-*cis-trans;* 3, 12-*trans-trans;* 4, 13-*trans-trans;* 5, 16-*cis-trans*; 6, 9-*cis-trans;* 7, 16-*trans-trans;* 8, 9-*trans-trans.*

between petroleum ether (bp 40-60 C) and aqueous ethanol and purified by chromatography on Hiflosil as previously described for the hydroperoxides from autoxidation of methyl linoleate (1).

The mixture appeared as a single component on thin layer chromatography (silicic acid, developed with 30:70 ether:hexane) when detected by UV absorption, ferrous thiocyanate spray, and chromic acid charring. Its UV spectrum consisted of a single absorption band at 235 nm. Iodometric titration (3) of the mixture yielded a hydroperoxide to diene absorption ($\epsilon_{235\ nm}$ = 24,000 assumed) ratio of 1.03. Reduction ($NaBH_4$) to the mixture of methyl hydroxylinolenates was carried out as previously described (5).

Methyl 9-hydroperoxy-*trans*-10,*cis*-12,*cis*-15-octadecatrienoate and methyl 13-hydroperoxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate were prepared using tomato and soybean lipoxygenases respectively as previously described for the oxidation of linoleate derivatives (4,5). When reduced to the methyl hydroxylinolenates, both preparations yielded a single isomer (the corresponding 9- and 13-hydroxy compounds) of over 90% isomeric purity (determined by HPLC).

Reduction to Methyl Hydroxystearates

The individual components of methyl hydroxylinolenate collected from HPLC of the mixture were reduced to the corresponding methyl hydroxystearates as previously described (5).

The mass spectra of the methyl hydroxystearates were examined for fragmentation ions resulting from isomers with the hydroxyl group in positions 8 to 16 (6). Only ions corresponding to isomers 9, 12, 13, and 16 were present to any appreciable extent (>30% of the base peak); the ions due to other isomers were in every case less than 1% of the base peak. The relative proportions of the intensities of the ions with m/e = 155, 197, 211, 253 (the 9, 12, 13, 16 isomers, respectively) from each component are listed in Table I.

RESULTS

In contrast to methyl linoleate hydroperoxides, the mixtures of the pure hydroperoxides obtained from the autoxidation of methyl linolenate was not resolved under the conditions of HPLC used but appeared as one composite peak. However, following reduction to the hydroxylinolenates, the mixture was resolved into eight major components (Fig. 1). The fact that the components are isomers of methyl hydroxylinolenate was confirmed by their individual UV spectra, all of which consisted of a single absorption band in the region 232-236 nm and by their IR spectra which were identical, apart from variations (see below) in

TABLE I

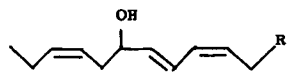
Ultraviolet (UV) and Infrared (IR) Spectral Data of Components of Methyl Hydroxylinolenate and Mass Spectrometry (MS) Data of the Derived Hydroxystearates

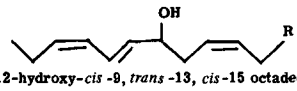
Component	% Composition (peak areas)[a]	UV (in ethanol) λ_{max} in nm	IR (in CCl_4) ν_{max} in cm^{-1}	MS of hydroxystearates (relative proportion of ions)			
				m/e =	155	197	211	253
				Isomer	9	12	13	16
1	10.2 ± 1.5	233	950, 989		–	5	95	–
2	8.2 ± 1.4	233	951, 990		–	94	6	–
3	1.9 ± 0.4	232	992		–	100	–	–
4	2.3 ± 0.4	232	992		–	4	96	–
5	38.1 ± 2.6	236	951, 989		10	–	–	90
6	30.0 ± 1.0	236	951, 988		100	–	–	–
7	5.8 ± 0.4	232	991		30	–	–	70
8	3.4 ± 0.5	232	992		100	–	–	–

[a]Average of three autoxidation experiments.

the region 950-1000 cm^{-1}, with that of a sample of methyl 13-hydroxy-*cis*-9,*trans*-11,*cis*-15-octadecatrienoate. Acid dehydration (7) of the individual isomers in ethanol solution yielded in every case the spectrum of a pure tetraene. Based on the value (7) of 49,200 for the molar extinction coefficient of the mixture of geometrical isomers of tetraenes, the yields of the conversion fall within the range 100 ± 15%. The geometrical and positional isomerism of each isomer was established by its IR spectra in the region 950-1000 cm^{-1} and by the mass spectra of the corresponding hydroxystearates (Table I). The mass spectra indicated that each component consisted essentially of one single positional isomer with a small degree of contamination from adjacent isomer(s). The apparently low isomeric purity of component 7 (a 16-isomer) is possibly due to the fact that it is a component positioned between two 9-isomers and that the 9-isomer gives rise to ions with higher intensity (approximately threefold) (6) than the 16-isomer. The purity of component 7 is therefore higher than that indicated by its mass spectrum. The geometrical isomerism of the double bonds that are in conjugation in each isomer was designated by analogy with the *cis-trans* and *trans-trans* isomers of methyl linoleate hydroperoxide (1) using the 950-1000 cm^{-1} region of the IR spectrum, i.e., a *cis-trans* isomer has two bands (950 and 989 cm^{-1}) while a *trans-trans* isomer has a single band (992 cm^{-1}). The absence of an absorption band at 970 cm^{-1} in their IR spectra confirmed that the double bond that is not in conjugation is, in each case, in a *cis* configuration. This allows the isomers 1-8 (Fig. 1) to be assigned the following structures:

R = $-(CH_2)_6COOMe$

1.  (13-*cis-trans*)
methyl 13-hydroxy-*cis*-9, *trans*-11, *cis*-15 octadecatrienoate

2.  (12-*cis-trans*)
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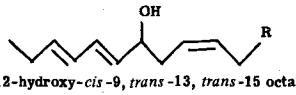
3.  (12-*trans-trans*)
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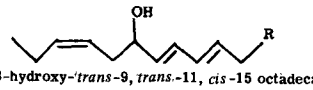
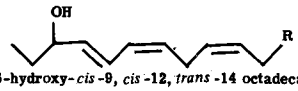
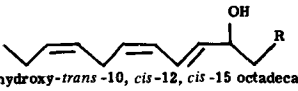
4.  (13-*trans-trans*)
methyl 13-hydroxy-*trans*-9, *trans*-11, *cis*-15 octadecatrienoate

5.  (16 *cis-trans*)
methyl 16-hydroxy-*cis*-9, *cis*-12, *trans*-14 octadecatrienoate

6.  (9-*cis-trans*)
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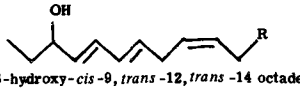
7.  (16-*trans-trans*)
methyl 16-hydroxy-*cis*-9, *trans*-12, *trans*-14 octadecatrienoate

8.  (9-*trans-trans*)
methyl 9-hydroxy-*trans*-10, *trans*-12, *cis*-15 octadecatrienoate

The identities of isomers 1 and 6 were further established by co-injection of authentic samples obtained from lipoxygenase oxidations. For convenience, the isomers of methyl hydroxylinolenate and methyl linolenate hydroperoxide are also designated by the position of the hydroxyl or hydroperoxy group and the geometrical isomerism of the double bonds that are in conjugation (e.g., 13-*cis-trans* etc.).

The average percentage composition of the peak areas of the components obtained from three separate autoxidation preparations are shown in Table I. However, there was insufficient material to enable a gravimetric determination of the molar extinction coefficients (ϵ-values) of all the components and thus any quantitation of the amounts of the components would require assumptions concerning their ϵ-values, especially the differences between *cis-trans* and *trans-trans* forms. The inaccuracies that arise from such assumptions are, however, minimized if not removed by summing the areas of both forms of a positional isomer. The relative proportions of the four positional isomers thus obtained were: 9-isomer, 33.4 ± 1.0; 12-isomer, 10.1 ± 1.4; 13-isomer, 12.5 ± 1.5; and 16-isomer, 43.9 ± 2.6.

DISCUSSION

Despite its complexity, the mixture of methyl hydroxylinolenates was separated into its constituent components by chromatography on Partisil-5. The components may be divided into two types, viz., the "outer" and "inner" isomers depending on whether the hydroxyl group is positioned outside the system of the three

double bonds (9 and 16 positional isomers) or whether it is placed within the double bond system so that it is sandwiched between a conjugated diene and an allyl function, i.e., it occupies an allylic and homo-allylic position (12- and 13-isomers). The two types are well separated by chromatography with the "inner" isomers having the shorter retention times. Analysis of the components confirmed the previous observation (2,6) that only four positional isomers (9, 12, 13, and 16) were formed from autoxidation of methyl linolenate. Like the autoxidation of methyl linoleate, two major geometrical isomers of each positional isomer were formed, viz., the *cis-trans* and the *trans-trans*. In the case of linoleate oxidation, the *cis-trans* isomer has the *trans* double bond adjacent to the hydroperoxide group. That this is also the case in linolenate autoxidation was indicated by cochromatography of lipoxygenase-produced isomers which have this configuration.

There are, however, differences between linoleate and linolenate autoxidation. Unlike linoleate oxidation, which yields equal proportions of 9 and 13 positional isomers (1), the relative proportions of the four positional isomers obtained from linolenate autoxidation are not equal but favor the 9 and 16 (i.e., "outer") isomers by a ratio of over 3 to 1. Frankel et al. (2) also obtained higher proportions of 9 and 16 isomers in their degradation studies. They attributed this either to the preferential attack of oxygen at positions 9 and 16 or to the higher rate of decomposition of the 12- and 13-hydroperoxides. The latter possibility can be discounted since we found that the 9 and 13 methyl linolenate hydroperoxides (obtained from lipoxygenase oxidations) decompose at similar rates. We have also ruled out the further possibility that the distribution of isomers observed is a result of the isomerization (8) of hydroperoxides after their formation; the pure 9- and 13-isomers did not undergo appreciable isomerization when heated at 40 C for 16 hr (Chan and Levett, unpublished data). Autoxidation of methyl linolenate is therefore regioselective, oxygenation occurring preferentially at positions 9 and 16 to favor the formation of "outer" isomers. The distribution of geometrical isomers is also significantly different for linolenate autoxidation which yields proportions of *cis-trans* isomers substantially higher than those of linoleate autoxidation (1).

The "inner" and "outer" isomers of methyl hydroxylinolenate also have minor differences in their UV spectra. While the "outer" isomers (9- and 16-) have a substantial difference in the wavelength of the absorption maxima of *cis-trans* (236 nm) and *trans-trans* (232 nm) isomers as is the case with the corresponding linoleate derivatives (1), the difference is much less marked for the "inner" isomers (12- and 13-) (Table I). This may be a consequence of the fact that the hydroxyl group is in a homoallylic position as well as adjacent to a conjugated diene in an "inner" isomer.

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Effect of Extended Hypocholesterolemic Drug Treatment on Peripheral and Central Nervous System Sterol Content of the Rat

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ABSTRACT

Extended treatment of developing or adult rats with a variety of hypocholesterolemic drugs has shown that both the central nervous system (CNS) and peripheral nervous system (PNS) sterol content could be affected in both age groups by this type of treatment. Treatment of developing animals was begun at 5 days of age and continued for 45 days. Adult rats, 300 g at onset, were treated for 35 days. The influence of these drugs on PNS sterol composition has not been previously examined. Diazacholesterol administration caused an accumulation of desmosterol in the CNS and 7-dehydrodesmosterol and desmosterol in the PNS. Zuclomiphene induced a build-up of desmosterol in either tissue. Both of these drugs had a more pronounced effect on developing CNS and PNS than on adult CNS and PNS. Addition of AY-9944 or Triparanol to the zuclomiphene treatment of the developing animals reduced desmosterol accumulation but brought about a build-up of 7-dehydrocholesterol and 7-dehydrodesmosterol.

INTRODUCTION

Cholesterol is a major lipid component of the nervous system. Administration of hypocholesterolemic drugs to developing rats has aided investigation of how cholesterol is formed in the nervous system and the manner by which it is utilized. The present series of experiments (1-7) have had two objectives: first, to establish if the appearance of membraneous cytoplasmic inclusion bodies, which frequently form in the developing nervous system after treatment with these hypocholesterolemic agents (8,9), could be correlated with the specific sterol or sterols being accumulated; and second, by using one or a combination of hypocholesterolemic drugs, provide further delineation of the pathway of cholesterol biosynthesis in the nervous system. In this regard, previous studies involving the developing rat central nervous system (CNS) have demonstrated that zuclomiphene [*cis* isomer of 2-(p-chloro-1,2-diphenylvinyl)phenoxyl)-triethylamine] causes an accumulation of desmosterol and zymosterol (7) (Fig. 1); AY-9944, an accumulation of 7-dehydrocholesterol (4); AY-9944 in combination with zuclomiphene, a build-up of desmosterol, zymosterol, 7-dehydrocholesterol, and 7-dehydrodesmosterol (1,4); and AY-9944 plus zulcomiphene plus Triparanol, an accumulation of desmosterol, zymosterol, 7-dehydrocholesterol, 7-dehydrodesmosterol, and 5α-cholesta-7,24-dien-3β-ol (2,5). Radioactive tracer studies have indicated even more points of inhibition of sterol formation by these drugs (3-5).

All of the previous experiments described sterol alterations in animals up to 20 days of age and only changes in the CNS portion of the nervous system. In the development of the brain, however, there are two distinct peak periods of cholesterol deposition (10). The initial peak, at about 10 days of age, seems to represent maximal deposition of cholesterol in nonmyelin structure, while the second peak, around 30 days of age, apparently represents maximal formation of cholesterol for myelin synthesis (10-12). Our previous studies obviously involved only the initial period of sterol deposition involving nonmyelin sterol. In order to assess the effect of these drugs on the deposition of sterol during this latter period, the present studies extend the period of treatment to 50 days of age, beyond the maximal period of myelin cholesterol biosynthesis.

Because of the reduced amount of cholesterol being formed by the adult brain (13), the effect of hypocholesterolemic drugs on the CNS of this age group has been only sparingly tested with AY9944 and Triparanol (14-17). The data reported here indicate the effect of two other hypocholesterolemic agents, zuclomiphene and 20,25-diazacholesterol, on adult rat brain and spinal cord sterol formation. In addition, the effect of hypocholesterolemic drugs on sterol content and composition of the sciatic nerve of both developing and adult animals has been measured for the first time. A preliminary report of this work has been presented (18).

MATERIALS AND METHODS

Developing rats (Wistar strain, both sexes) were given four different regimens of hypocholesterolemic drugs by intraperitoneal injection: 20,25-diazacholesterol (30 mg/kg body

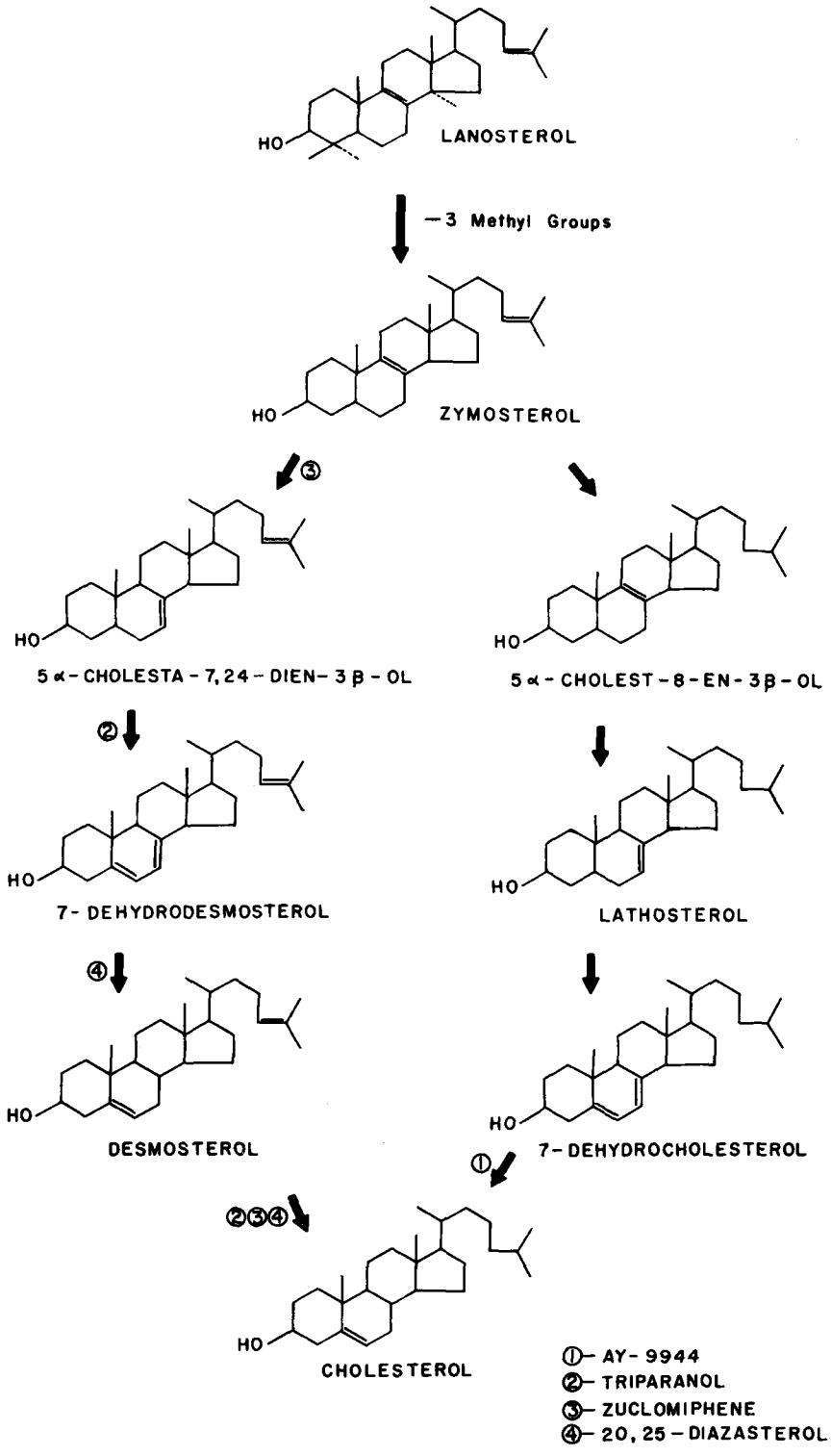

FIG. 1. Previously determined sites of inhibition of central nervous system (CNS) sterol biosynthesis by the four individual hypocholesterolemic drugs studied are indicated (7-9).

wt) dissolved in saline, zuclomiphene citrate (30 mg/kg) in propylene glycol:water (1:1, v/v), zuclomiphene (30 mg/kg) plus AY9944 (3 mg/kg) in propylene glycol:water (1:1, v/v), or zuclomiphene (30 mg/kg) plus Triparanol (30 mg/kg) plus AY9944 (3 mg/kg) in propylene glycol:water (3:1, v/v). Treatment was initiated at 5 days of age. Animals were injected twice weekly and received a total of 13 injections before sacrifice at 50 days of age. Controls received either saline or propylene glycol:water (3:1, v/v). No difference in sterol content was noted for these two types of controls. Adult rats were given 20,25-diazacholesterol (30 mg/kg) or zuclomiphene (30 mg/kg) by intraperitoneal administration twice weekly for a total of 10 injections before sacrifice 35 days after initiation of treatment. The two sciatic nerves of each animal were combined for analysis.

Lipids were extracted by the method of Folch et al. (19). Extractions were taken to dryness under nitrogen, redissolved in ethyl acetate, and further fractionated by thin layer chromatography (TLC) and silver nitrate-impregnated TLC as described previously (3). Sterols were further chromatographed by means of gas liquid chromatography (GLC). A 3% OV-17 column, which was operated at 265 C, was utilized (3). Identification of sterol peaks was made by comparison to standard sterol retention times previously derived. Quantitation of peaks was by triangulation. 5α-Cholestane was used as an internal standard.

RESULTS

Of the four different types of drug treatment presented to developing animals, diazacholesterol administration had the most pronounced effect on brain cholesterol concentration (Table I). Zuclomiphene treatment prevented the conversion of only one-third of the brain's desmosterol to cholesterol. The addition of AY-9944 to the zuclomiphene treatment resulted in a substantial accumulation of 7-dehydrocholesterol, but reduced the desmosterol content to only trace amounts. Triparanol addition to the drug regimen again resulted in a build-up of Δ^{24}-sterol, but in the form of 7-dehydrodesmosterol not desmosterol. Total sterol content was slightly reduced with all types of drug treatment.

The pattern of sterol accumulation in the spinal cords of treated animals was similar to that seen in the brain (Table II). Zuclomiphene had a more pronounced effect on the build-up of desmosterol in the spinal cord than in the brain. The accumulation of 7-dehydro-

TABLE I

Sterol Content of Brain Tissue of Developing Animals Treated with Hypocholesterolemic Drugs and Corresponding Control Animals[a]

Sterol	Type of treatment											
	Control		Diazacholesterol		Zuclomiphene		Zuclomiphene + AY9944		Zuclomiphene + Triparanol + AY9944	
	(wt[b])	(%[c])	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)
Cholesterol	58.0 ± 5.21	100	5.42 ± 0.37	10.6 ± 0.86	36.0 ± 4.27	66.2 ± 5.83	30.8 ± 0.96	58.9 ± 0.85	33.1 ± 2.92	69.3 ± 4.26
Desmosterol	-	-	45.6 ± 2.02	89.4 ± 0.86	18.3 ± 3.19	33.8 ± 5.83	trace	-	trace	-
7-Dehydrocholesterol	-	-	-	-	-	-	21.5 ± 1.12	41.1 ± 0.85	11.3 ± 2.12	23.2 ± 4.92
7-Dehydrodesmosterol	-	-	-	-	-	-	trace	-	4.61 ± 0.60	9.37 ± 0.98
Total sterol	58.0 ± 5.21	-	51.0 ± 1.98	-	54.4 ± 3.66	-	52.3 ± 1.94	-	49.1 ± 1.36	-

[a]Values are ± standard deviation for four controls and three test animals in each instance. Sterols were separated by means of $AgNO_3$ thin layer chromatography and quantitated by gas liquid chromatography using 5α-cholestane as an internal standard.

[b]mg/g Dry wt tissue.

[c]% Of total sterol.

TABLE II

Sterol Content of Spinal Cord Tissue of Developing Animals Treated with Hypocholesterolemic Drugs and Corresponding Control Animals[a]

	Type of treatment									
	Control		Diazacholesterol		Zuclomiphene		Zuclomiphene + AY9944		Zuclomiphene + Triparanol + AY9944	
Sterol	(wt[b])	(%[c])	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)
Cholesterol	94.6 ± 5.90	100	10.4 ± 1.53	12.4 ± 1.36	49.8 ± 5.78	52.6 ± 5.71	35.5 ± 3.12	39.9 ± 1.67	39.3 ± 2.91	41.8 ± 1.21
Desmosterol	-	-	73.0 ± 1.78	87.6 ± 1.36	45.0 ± 5.58	47.4 ± 5.71	trace	-	trace	-
7-Dehydrocholesterol	-	-	-	-	-	-	46.3 ± 2.70	52.0 ± 1.28	39.1 ± 1.57	41.6 ± 0.57
7-Dehydrodesmosterol	-	-	-	-	-	-	7.17 ± 0.93	8.13 ± 1.42	15.6 ± 1.11	16.6 ± 1.01
Total sterol	94.6 ± 5.90	-	83.4 ± 3.25	-	94.8 ± 2.93	-	89.0 ± 4.50	-	94.0 ± 5.00	-

[a]Values are ± standard deviation for four controls and three test animals in each instance. Sterols were separated by means of $AgNO_3$ thin layer chromatography and quantitated by gas liquid chromatography using 5α-cholestane as an internal standard.

[b]mg/g Dry wt tissue.

[c]% Of total sterol.

TABLE III

Sterol Content of Sciatic Nerve of Developing Animals Treated with Hypocholesterolemic Drugs and Corresponding Control Animals[a]

	Type of treatment									
	Control		Diazacholesterol		Zuclomiphene		Zuclomiphene + AY9944		Zuclomiphene + Triparanol + AY9944	
Sterol	(wt[b])	(%[c])	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)
Cholesterol	63.6 ± 7.82	100	4.00 ± 0.15	6.17 ± 0.32	22.5 ± 3.67	33.9 ± 4.54	12.9 ± 1.00	20.3 ± 0.81	13.0 ± 1.69	20.2 ± 0.95
Desmosterol	-	-	47.2 ± 2.35	72.9 ± 1.57	43.8 ± 1.87	66.1 ± 4.54	-	-	trace	-
7-Dehydrocholesterol	-	-	-	-	-	-	36.6 ± 1.27	58.0 ± 3.70	29.4 ± 2.14	46.1 ± 2.25
7-Dehydrodesmosterol	-	-	13.6 ± 2.07	20.9 ± 1.78	-	-	13.7 ± 2.62	21.7 ± 3.36	21.6 ± 2.16	33.7 ± 1.72
Total sterol	63.6 ± 7.82	-	64.8 ± 4.48	-	66.3 ± 2.35	-	63.2 ± 2.63	-	64.0 ± 5.22	-

[a]Values are ± standard deviation for four controls and three test animals in each instance. Sterols were separated by means of $AgNO_3$ thin layer chromatography and quantitated by gas liquid chromatography using 5α-cholestane as an internal standard.

[b]mg/g Dry wt tissue.

[c]% Of total sterol.

sterols in the combination-drug treatment situation was also more extensive in the spinal cord.

The types of sterol present in the treated sciatic nerves were the same as the CNS, but the levels of noncholesterol sterols were generally higher (Table III). Unlike in the CNS, diazacholesterol caused an accumulation of 7-dehydrodesmosterol, as well as desmosterol, in this nerve of the peripheral nervous system (PNS). Desmosterol content of sciatic nerve derived from zuclomiphene treatment was twice that found in brain. The situation was similar for the 7-dehydrosterol content of sciatic nerve vs. brain when the treatment was that involving a combination of hypocholesterolemic drugs.

As would be expected, the effect of the hypocholesterolemic drugs on the sterol formation of the mature nervous system was less pronounced than that of the developing tissue (Table IV). Diazacholesterol was twice as effective as zuclomiphene in affecting an accumulation of desmosterol in the adult brain and spinal cord. The relative action of these drugs was the same in the sciatic nerve. As with the developing PNS, diazacholesterol brought about a build-up of 7-dehydrodesmosterol in the mature sciatic nerve, although at a much reduced level.

TABLE IV

Sterol Content of Brain, Spinal Cord, and Sciatic Nerve of Adult Animals Treated with Hypocholesterolemic Drugs and Corresponding Control Animals[a]

Sterol	Type of treatment															
	Control						Diazacholesterol						Zuclomiphene			
	Brain		Spinal cord		Sciatic nerve		Brain		Spinal cord		Sciatic nerve		Brain		Spinal Cord		Sciatic nerve	
	(wt[b])	(%[c])	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)	(wt)	(%)
Cholesterol	45.6 ± 2.29	100	83.0 ± 2.93	100	51.0 ± 1.25	100	38.6 ± 0.92	85.6 ± 4.48	71.6 ± 0.70	87.2 ± 2.54	43.5 ± 2.10	84.1 ± 2.76	41.2 ± 2.08	92.7 ± 1.59	80.7 ± 2.64	94.8 ± 0.53	48.8 ± 5.41	90.4 ± 2.26
Desmosterol	.	.	.	.	.	.	6.5 ± 2.23	14.4 ± 4.48	10.5 ± 2.36	12.8 ± 2.54	6.93 ± 1.43	13.4 ± 3.08	3.24 ± 0.58	7.30 ± 1.59	4.44 ± 0.60	5.20 ± 0.53	5.07 ± 0.79	9.57 ± 2.26
7-Dehydrodesmosterol	.	.	.	.	.	.	.	.	.	.	1.30 ± 0.65	2.47 ± 1.17	.	.	.	.	.	.
Total sterol	45.6 ± 2.29	.	83.0 ± 2.93	.	51.0 ± 1.25	.	45.2 ± 1.67	.	82.1 ± 2.05	.	51.7 ± 1.30	.	44.4 ± 1.51	.	85.1 ± 3.23	.	53.9 ± 5.60	.

[a] Values are ± standard deviation for three controls and three test animals in each instance. Sterols were separated by means of $AgNO_3$ thin layer chromatography quantitated by gas liquid chromatography using 5α-cholestane as an internal standard.

[b] mg/g Dry wt tissue.

[c] % Of total sterol.

DISCUSSION

Dvornik and Hill (17) demostrated a number of years ago that chronic administration of AY-9944 (6 to 12 months) to adult rats resulted in 7-dehydrocholesterol representing up to 50% of the total brain sterol. They were also able to show that giving a single oral dose of AY-9944 to adult rats brought about a two-thirds reduction in cholesterol-forming ability 2 hr after treatment, using labeled acetate incorporation in vivo as a means of measure (15). Short-term administration of AY-9944, Triparanol or diazacholesterol, however, changed the adult rat endogenous sterol content very little (14,16). Thirty-five days of treatment in the present study did produce higher levels of cholesterol precursors in the CNS than did previous studies. Diazacholesterol was more effective in the adult than was zuclomiphene. Effectiveness may be a measure of binding of the inhibitor to the enzyme, or it may be indicative of the respective drug's permeability into the CNS.

Because the developing brain has a high rate of cholesterol biosynthesis, the use of cholesterol inhibitors has always been more fruitful in this type of animal. The question does arise,

however, if the animal is treated through the period of most active myelination (5-50 days), do the levels of cholesterol precursors in the nervous system remain as high as they were during the height of this active period? Fumagalli et al. (20) demonstrated that when treatment of rats with AY-9944 or diazacholesterol was terminated at 21 days of age, there was a rapid reduction of 7-dehydrocholesterol and desmosterol content in the CNS. Suzuki et al. (8), however, have indicated that an animal treated from 5 to 60 days of age with AY-9944 presumably had a constant level of 7-dehydrocholesterol within the brain from about 25 days of age until the end of the experiment. We were not able to make the same observation. Although the drug dosage of zuclomiphene and zuclomiphene plus AY-9944 were slightly reduced from our earlier more acute experiments (1,7), the experiments involving zuclomiphene plus Triparanol and AY-9944 were at the same level as with the shorter term experiments (5,6). In all three instances, the level of brain and spinal cord cholesterol precursors was lower at 50 days of age than it was at 20.

The marked effect of the addition of AY-9944 to the zuclomiphene-induced sterol profile is difficult to explain. The reduced level of desmosterol and large increase of 7-dehydrocholesterol were seen in the short-term experiments as well (1,7). In the present study, desmosterol accumulation was completely negated by addition of AY-9944. Since the relative activities of the respective enzymes involved sterol Δ^{24} reductase (21) and sterol Δ^{7} reductase (22), are both maximal at ca. 8-12 days of age in the rat and recess rapidly to basal levels around 20 days of age, one would theorize that neither would dominate and, therefore, the use of inhibitors of both enzymes would result in the accumulation of 7-dehydrodesmosterol. Another factor must be involved because this is not the actual situation.

The effect of hypocholesterolemic agents on PNS sterol composition had not been previously examined. Basically, the sterol deposition in the PNS and CNS was the same under analogous conditions. The accumulation of 7-dehydrodesmosterol as a result of diazacholesterol treatment was a new observation, however, and may reflect a basic difference in the manner which the PNS Schwann cell and the CNS astrocyte and oligodendroglia form cholesterol. At present, very little is known about PNS sterol biosynthesis. It is possible that further utilization of diazacholesterol may aid in establishing how sterol synthesis proceeds in the peripheral nervous system.

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A Mass Spectrometric Survey of Some Biologically Important Lipids

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ABSTRACT

The mass spectra of lipids are reviewed with emphasis on characteristic fragmentation patterns of each general class of compounds. Recent developments in the area of field ionization and field desorption mass spectrometry are discussed relative to each class of lipids.

INTRODUCTION

Mass spectrometry is one of the many analytical instruments available to lipid researchers (1-3). In recent years, mass spectrometric applications to problems in the biological, environmental, and medical sciences have assumed increased significance, and the whole field is undergoing remarkable expansion. In just two years (1972 and 1973), over twenty thousand publications appeared which involved mass spectrometry. Many equivalent problems in biology, ecology, and medicine lend themselves to qualitative or quantitative mass spectrometry or the combination of gas chromatography and mass spectrometry (GC-MS). GC-MS is particularly useful for the studies of lipids because of the ease with which complex mixtures can be separated and identified. GC-MS is capable of detecting, separating, and characterizing organic compounds in extremely small samples. Mass spectrometry is one of the few techniques which can provide definite structural information from minute quantities of material.

In general, any compounds which are volatile enough for gas chromatographic separation are sufficiently volatile for mass spectrometric analysis. The combination of the two techniques is a powerful analytical tool and has greatly aided scientists in the identification of compounds present in complex natural and synthetic mixtures. Compounds which are not sufficiently volatile for GC-MS analysis can frequently be converted to volatile derivatives for electron-impact mass spectral studies. A relatively new technique, field desorption mass spectrometry (FDMS), has emerged which shows promise of producing ions even from compounds of high molecular weight and low volatility (4). There is much widely dispersed information available on the mass spectrometry of the classes of lipids considered here. This article is an attempt to provide a reasonably comprehensive review of the salient features of fragmentation patterns of lipids in the hope that it will be of use to those involved in the investigation of natural products as a practical introductory guide.

FATTY ACIDS

Relatively few studies have been done on the electron impact mass spectra of free fatty acids, because their methyl esters have greater volatility and undergo less thermal degradation than do the free acids. The pioneering studies on the mass spectra of the methyl esters of fatty acids were done by Ryhage (5) and Ryhage and Stenhagen (6,7). More recently, the mass spectra of fatty acids and complex lipids have been reviewed by Sun and Holman (8) and Odham and Stenhagen (9). The mass spectra of methyl esters of long normal chain acids are similar in appearance. The spectrum of methyl stearate typifies the general appearance of the mass spectra of this class of compounds (9). The most prominent and informative peaks are due to ions containing oxygen (Fig. 1). Many of these ions belong to a series of the general formula $[\text{-}(CH_2)_nCOOCH_3]^+$ and contain an intact methoxy carbonyl group. A periodicity is observed in these peak intensities and the peaks at m/e 87 (n = 2), m/e 143 (n = 6), and m/e 199 (n = 10), which are separated by four methylene groups, have a greater relative abundance than the other peaks of this series. The spectrum contains three additional significant peaks containing oxygen: (a) the molecular ion, (b) the acylium ion, and (c) the rearrangement ion of m/e 74. Typical of carbonyl compounds, methyl esters of long chain acids undergo a cleavage of the alkoxy group from the molecular ion to yield the relatively stable acylium ion, with m/e = M-31 (Fig. 2). Like other carbonyl compounds, esters of fatty acids undergo a β-cleavage with the transfer of a γ-hydrogen, which is referred to as the McLafferty rearrangement and results in the base peak of the normal chain methyl esters in the range C_6-C_{26} with m/e 74 (Fig. 3). Deuterium-labeling studies on methyl esters with a $\text{-}CD_2\text{-}$ group at different distances from the carbonyl group are in agreement with the above mechanism, and a shift in the base peak to m/e 75 is observed in the case of 4,4-dideuteroeicosanoate (11). An example of an unusual fragmentation pathway in methyl

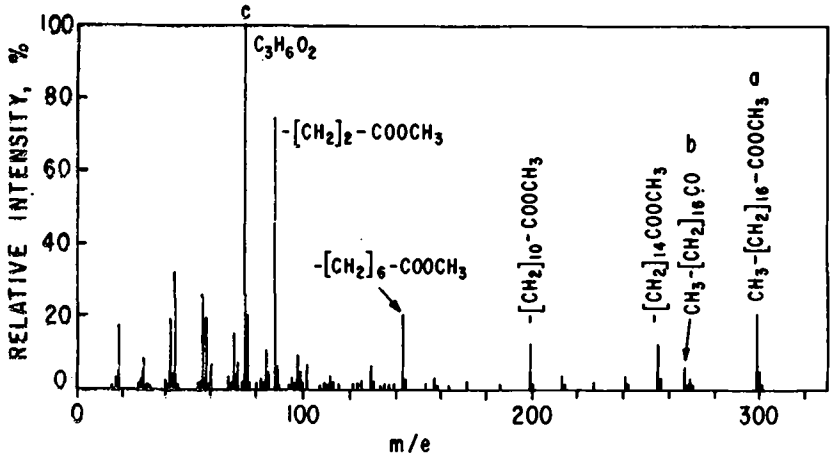

FIG. 1. The mass spectrum of methyl stearate. Three major peaks are: (a) the molecular ion, (b) the acylium ion, and (c) the ion of m/e 74. (10).

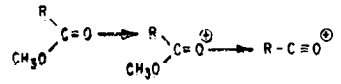

FIG. 2. The acylium ion. For methyl esters, this peak has m/e of M-31.

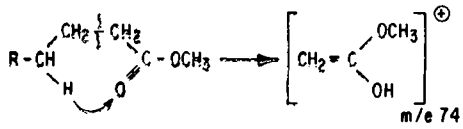

FIG. 3. The rearranged ion of m/e 74, also called the McLafferty rearrangement. This ion is due to β-cleavage with γ-hydrogen transfer.

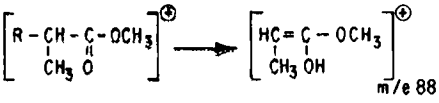

FIG. 4. Substitution by an α-methyl gives a McLafferty ion at m/e 88.

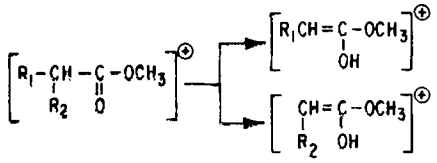

FIG. 5. In α-branched fatty acid esters, the McLafferty rearrangement can occur in either of two ways.

esters of long normal chain acids is the loss of 43 mass units. Although one might expect this represents the loss of the end three carbon atoms of the alkyl chain, an extensive investigation by Ryhage and Stenhagen using deuterium-labeled molecules has demonstrated that the majority of the M-43 fragments are due to the elimination of C_2, C_3, and C_4 from the molecular ion, accompanied by a hydrogen transfer (12).

The presence of a branch in the hydrocarbon chain produces a characteristic change in the mass spectrum. A methyl group on the carbon atom next to the carbonyl group (α-methyl) shifts the base peak to m/e 88. This illustrates that the α-carbon atom with the attached methyl group remains in the rearranged ion (Fig. 4). When the α-positioned side chain is an ethyl or larger alkyl group, the McLafferty rearrangement (2,3-cleavage with γ-hydrogen transfer) can occur in two different ways as shown in Figure 5. In general, the esters of the series $CH_3-(CH_2)_n-CH(R)-(CH_2)_m-COOCH_3$ show preferential breaking of the bonds at the tertiary carbon atom. In the case of R = CH_3, two prominent peaks are observed due to the methoxy carbonyl ions (A) and (B) (Fig. 6). The methoxy carbonyl ion representing the ion (B-R) gives a very small peak. A peak of high relative intensity occurs due to the loss of methanol from (B), and is probably a ketene type ion (C). When the site of the branching in the hydrocarbon chain is at some distance from the carbonyl group (M > 6), the simple cleavage ion (A) is accompanied by rearrangement ions corresponding to (D) and (E). There are two positions of a methyl branch in the hydrocarbon chain that warrant special attention. One of these is a methyl group at position 6 which gives a high peak at M-76, not found in the spectra of the 5- and 7-methyl-substituted compounds. The second special case is the methyl

ester of an iso-acid containing a terminal isopropyl group (methyl branch at position ω-1) which gives a spectrum that is very similar to the normal chain isomer. A small peak due to the loss of methanol, water, and a methyl group, M-65, that is not present in the normal chain isomer, is present in the spectrum of the iso-acid. The iso-acids can be recognized through comparison of the mass spectra of the corresponding alcohols (7).

Polymethyl-substituted esters in which the tertiary carbon atoms are separated by a number of methylene groups follow the same general fragmentation scheme as the monomethyl-substituted esters. When two tertiary carbon atoms are next to each other, cleavage occurs very readily between the tertiary carbons.

The mass spectra of α-monosubstituted and α,α-disubstituted fatty acid esters have been investigated by Foglia, et al. (13). Recent study has shown that in α,α-disubstituted esters the M-59 ion, a trialkyl carbonium ion due to the loss of the methoxy carbonyl group, is a prominent ion. This illustrates an unusual situation in the spectra of fatty acid esters in which there is retention of the positive charge by the hydrocarbon fragment at high m/e. This is accompanied by a decrease in the relative abundance of the molecular ion. Disubstitution by ethyl or larger alkyl groups at the α-position provides three different routes of γ-hydrogen transfer and thus three routes for the McLafferty rearrangement. Although all of the rearrangements occur, the hydrogen transfer from the larger alkyl group is favored

The case of methyl-3,3-dimethyl heneicosanoate similarly shows a peak at M-73 due to a trialkyl carbonium ion which results from 2,3-cleavage (14). This further illustrates that

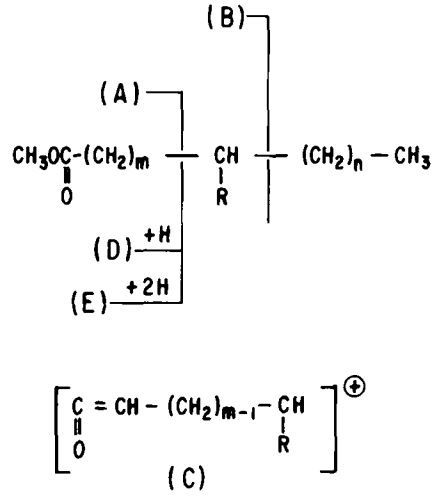

FIG. 6. A generalized scheme for the fragmentation of branched fatty acid ester. Cleavage occurs readily α- to the branch.

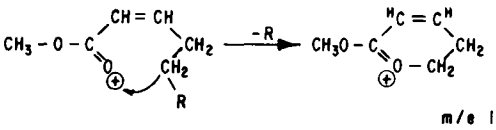

FIG. 7. The characteristic peak at m/e 113 found in α,β-unsaturated fatty acid esters.

esters containing a quaternary carbon atom show a very strong tendency to cleave the bonds of the quaternary carbon atom and to retain the positive charge at the trialkyl carbon fragment.

The mass spectra of α,β-unsaturated normal chain fatty acid esters show a charateristic peak

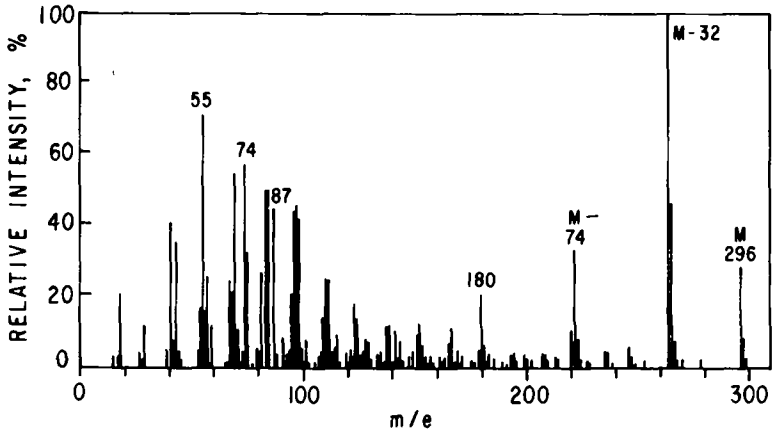

FIG. 8. The mass spectrum of methyl oleate, a monounsaturated fatty acid ester. A prominent peak to note is the M-32. (16).

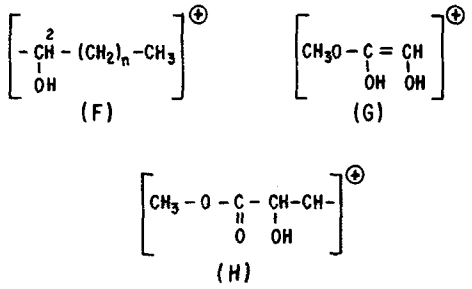

FIG. 9. Characteristic ions in the mass spectrum of hydroxy, fatty acid methyl esters.

at m/e 113 due to ions former on 5,6-cleavage. McLafferty has proposed that this ion exists in cyclic form (15) (Fig. 7). The mass spectra of positional isomers at 6,7- for further removed from the carbonyl group are very similar to the mass spectra of methyl oleate; therefore, it is not possible to deduce the position of the double bond or the geometrical configuration by mass spectrometry alone. The M-32 peak due to the loss of the elements of methanol is a prominent peak in the mass spectra of monoenoic esters, and in the case of methyl oleate constitutes the base peak of the spectrum (17) Fig. 8). Two other abundant peaks characteristic of monoenoic ester spectra are the M-74 and M-116 peaks, which are hydrocarbon type ions formed by 2,3- and 5,6-cleavage, respectively. The spectra of esters with more than one double bond are characterized by an increase in the abundance of the molecular ion.

The determination of the position of one or more double bonds in an unsaturated fatty acid ester is possible by chemical derivatization.

One of the classical methods of location of the position of the double bond in a long chain compound is oxidative degradation. GC-MS can be used for the subsequent isolation and identification of the reaction products which consist of a saturated fatty acid and a dibasic acid (14). Another approach is the use of deuterohydrazine, which introduces two deuterium ions at the site of the double bond, and the position of the double bond was deduced by comparison of the deuterated and nondeuterated ester spectra although the authors concluded that this was not a satisfactory method (16).

Andersson and Holman (18) have recently approached the location of the double bond in fatty acid esters using MS by derivatization with pyrrolidine, since the amide group has a charge stabilization effect upon the fatty acid moiety. When comparing the mass spectra of the methyl esters of petroselinic acid (Δ^6-18:1) and vaccenic acid (Δ^{11}-18:1), it was noted, as stated previously, that the spectra were very similar to each other and the spectra of other isomeric monounsaturated fatty acid esters. Clear differences were observed in the pyrrolidide derivatives of the two acids, and from the mass spectra of all isomers of 18:1 from Δ^5-Δ^{15}. If an interval of 12 mass units, instead of the normal 14, appeared between the most intense peaks of the clusters of fragments containing n and n-1 carbon atoms, the double bond could be located between carbon atoms n and n+1 in the molecule. The pyrrolidide derivative may offer several advantages for the structural analysis of fatty acid esters. The derivative is easily prepared, quantitatively, in one step on the nanogram scale. Because only one fragmentation pattern occurs, the rules for interpreting mass spectra of pyrrolidides apply to a wide range of isomers of monoenoic acids, branched, deuterated, acetylenic, cyclopropane, and other fatty acids. The pyrrolidide derivative permits GC-MS analysis of a wide range of structures occurring in a single sample, thus minimizing purification steps and derivatization procedures. Therefore, the use of the pyrrolidides may become a general analytical tool. One limitation, however, may be that the peaks of interest in this approach are aften less than 10% in relative abundance and could be difficult to interpret.

The mass spectra of hydroxy esters in the series CH_3-$(CH_2)_n$-CH(OH)-$(CH_2)_m$-$COOCH_3$ show a weak molecular ion, and in some cases the molecular ion is absent from the spectrum. For hydroxy esters with $m>5$, an abundant peak due to the loss of the hydrocarbon chain beyond the carbon atom containing the hydroxy group is observed. A prominent ketene type ion is derived from the ion previously mentioned through loss of the methoxy group and a methylene group. α-Cleavage at the ester side of the carbon bound to the hydroxy group, with loss of the hydroxy group and hydrogen transfer, occurs in esters with $m>4$. The loss of the elements of water and methanol from the molecular ion gives rise to a peak at M-50, due to ketene type ions. A prominent peak in 2-hydroxy esters is due to the cleavage between carbon atoms 1 and 2 (Fig. 9) which gives rise to "alkanol" ions of M-59 (F). In addition, large characteristic peaks due to the McLafferty rearrangement ion at m/e 90 (G) and the ion of m/e 103 (H) are observed, which are analogous to the ions of m/e 74 and 87, respectively, which appear in the spectrum of the unsubstituted methyl ester (19).

The use of trimethylsilyl (TMS) derivatives in establishing the position of the hydroxy groups in hydroxy fatty acid esters has the advantage in that cleavage α to the TMS group

gives rise to intense peaks, which allows assignment of the hydroxy position (20). For the general case, CH_3-$(CH_2)_n$-CH(OTMS)-$(CH_2)_m$-$COOCH_3$ α-cleavage with charge retention or the $[CH_3$-(CH_2)-CH(OTMS)$]^+$ fragment is the key peak in assignment of the hydroxy position. Di-TMS derivatives of diols in the series CH_3-$(CH_2)_n$-CH(OTMS)-CH(OTMS)-$(CH_2)_m$-$COOCH_3$) give predominant cleavage between the trimethylsiloxy groups producing two fragments $[CH_3$-$(CH_2)_n$-CH(OTMS)$]^+$ and $[$-CH(OTMS)-$(CH_2)_m$-$COOCH_3]^+$ with the positive charge retained almost equally on the two fragments (21). Although the molecular ion is not generally present in these mass spectra, peaks due to M-15 and M-30 clearly indicate the molecular weight. TMS derivatives have also been successfully applied to elucidating the position of double bonds. The TMS derivatives are prepared by oxidation with osmium tetroxide, followed by silylation of the obtained diol. The characteristic fragmentation of the di-TMS derivative of the diol establishes the position of the original double bond (22).

The methyl ester of an oxo-acid (Fig. 10) shows prominent fragmentation at the α- and β-positions with respect to the oxo-group. α-Cleavage gives rise to ions (I) and (J), whereas β-cleavage with the loss of the oxo-group gives (K). β-Cleavage gives rise to ions (L) and (M), with the oxo-group remaining in the ionized fragment accompanied by the transfer of one hydrogen. Ion (M) also fragments further to give a ketene type ion through the loss of 32 mass units, which makes a prominent appearance in oxo-ester spectra. In addition, the molecular ion and acylium ion (M-31) give abundant peaks and in conjunction with the α- and β-cleavage fragments already mentioned allow for the elucidation of the structure of the ester (19). Special cases are found in the 2- and 3-oxo esters. The bond between juxtaposed keto-groups in the case of the 2-oxo ester is a site where fragmentation readily occurs (Fig. 11). Acylium ions of M-59 are formed so easily that other ions containing oxygen have a low relative intensity. The molecular ion and an ion of m/e 103 (N), due to 3,4-cleavage and the transfer of two hydrogens, appear in the spectra. In the case of the 3-oxo or β-keto ester, a large peak is observed at m/e 58, apparently due to an ion containing carbon atoms 2, 3, and 4 of the original molecule in which two hydrogens have been transferred. This ion probably exists in the enol form (O). Two ions at the high mass end of the spectrum are the result of the expulsion of carbon atoms in the hydrocarbon chain. A peak at M-42 is due to the loss of

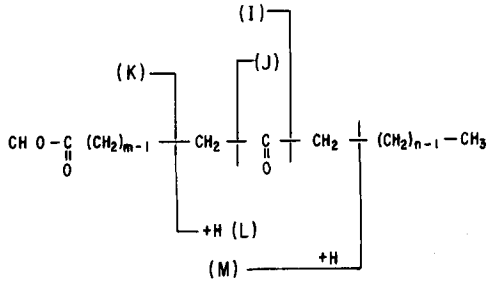

FIG. 10. A generalized fragmentation pattern for oxo-fatty acid esters. Both α- and β-cleavage with respect to the carbonyl moiety are observed.

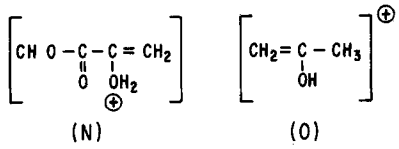

FIG. 11. Ions of importance in 2-oxo (N) and 3-oxo (O) fatty acid methyl esters.

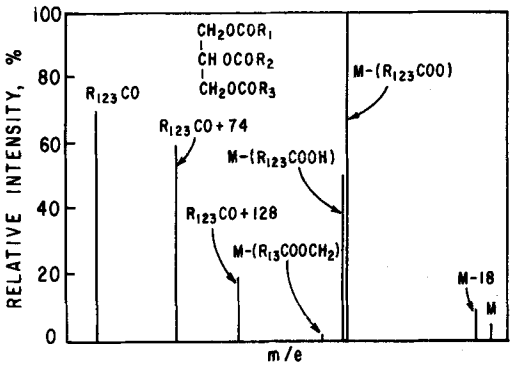

FIG. 12. The generalized mass spectrum pattern for a triglyceride. (23).

ketene containing carbon atoms 2 and 3, while the peak at M-58 is due to the expulsion of an acetone moiety containing carbon atoms 2, 3, and 4.

Methoxy-substituted esters give prominent characteristic peaks due to α-cleavage to the substituted carbon. Epoxy derivatives also undergo α-cleavage relative to the spoxy substituent which gives rise to abundant peaks. It is of importance to note that cleavage of the oxirane ring on the ester side is accompanied by the loss of two hydrogens (19).

TRIGLYCERIDES

In general, the mass spectra of triglycerides are characteristic and the important peaks are readily interpreted in terms of triglyceride structure (23-25). Barber et al. (23) have shown

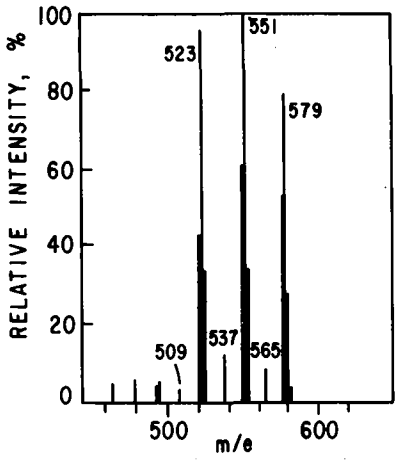

FIG. 13. A section of the mass spectrum of 1-myristo-2-stearo-3-palmitin showing the M-(RCOOH) and M-(RCOOCH$_2$) peaks which are useful for structure determination. (23).

that even the spectra of a mixed triglyceride (i.e., in which the fatty acids esterified to glycerol are different) have certain features which make it possible to distinguish between the different isomers and isologs. The base peak in the mass spectrum of a triglyceride is usually the M-RCOO due to the loss of an acyloxy group from the complete molecule (Fig. 12). In the case of a mixed triglyceride, a peak is observed from the loss of each acyloxy group in the molecule and therefore, this peak is very important for structural elucidation. The positively charged acyl ion, RCO, is generally the second most abundant peak in a triglyceride spectrum. In a mixed triglyceride, there occurs an acyl ion corresponding to each fatty acid present in the molecule. The peak occurring one mass unit lower than the base peak at M-RCOOH is of interest because it is the precursor of many of the ions of lower mass. Associated with each of the M-RCOO peaks, there are additional peaks of lower relative abundance 14 mass units lower. In a mixed triglyceride, this series of peaks is of great importance in discriminating positional isomers. The peak that arises from the loss of RCOOCH$_2$ at the 2-position is significantly smaller than when this fragment is lost from the 1- or 3-position. In the spectrum of 1-myristo-2-stearo-3-palmitin, this characteristic fragmentation pattern is clearly illustrated (Fig. 13). The peak at m/e 509 due to the loss of the stearic fragment is less than one-third the relative abundance of the peaks at m/e 565 and 537, due to the loss of the myristic and palmitic fragments, respectively.

A set of peaks occurring at 74 and 128 mass units higher than each acyl ion are characteristic of an acid attached directly to the glycerol skeleton and establishes this structural feature. A prominent metastable ion is observed corresponding to the M→(M-18) transition, and indicates that the peak which is always observed at M-18 is largely due to ionic fragmentation and not to thermal degradation. The complete structural analysis of a triglyceride containing straight chain fatty acids is apparently possible and has considerable advantages in economy of time and material over the usual methods of glyceride analysis.

One drawback to this approach that may present itself is the fact that the M-RCO$_2$CH$_2$ peaks, which are used to assign the relative position of the fatty acids on the glycerol skeleton in a mixed triglyceride, are less than 10% relative intensity and may be difficult to interpret.

The quantitative analysis of triglyceride mixtures has been accomplished by Hites (26) by placing the sample directly into the ion source, via the direct probe, and the sample is fractionated by molecular distillation, which is a function of molecular weight. The intensities of the molecular ion and M-18 peak are measured, and the analysis can be completed in as little as 20 min with only 100 to 500 μg of sample. This method cannot distinguish, however, between isomers and isologs of mixed triglycerides. A combined system of gas chromatograph and mass spectrometer by Murata and Takahashi (27) permits rapid analysis of triglyceride samples. A sample is first separated on the basis of carbon number by gas chromatography and the constituent fatty acids are identified by mass spectrometry.

Limitations in obtaining the electron impact mass spectra of triglycerides are the result of the low volatility and high molecular weight of this class of compounds. These limitations make it difficult to introduce triglycerides into the mass spectrometer as a vapor and even more difficult to remove them from the ion source. Ions are usually produced in a mass spectrometer by bombarding the sample in the vapor state with high energy electrons (70 eV) which produce the positive ions used for analysis. The electron impact method, however, gives the positive ion a high vibrational energy which gives rise to further fragmentation and the considerable number of peaks found in the standard mass spectrum. Another approach to ion production is the field ionization source. In the field ionization source, a high voltage potential is applied between a sharp edge and a slit, and the resulting electric field that is produced is about 100 million volts per centimeter. Electrons are removed from the vapor molecules by a quantum mechanical tunneling

mechanism which produces positive ions with low vibrational energy and consequently very little fragmentation (28). The molecular ions formed in field ionization have little excess energy and often appear as the most abundant peak in the spectrum, even when no molecular ion is observed in the electron impact spectrum. The fewer and relatively smaller fragment ions make the identification and measurement of molecular ion intensities easier and more reliable (29).

Rohwedder (30) has reported the field ionization spectra of methyl stearate, methyl oleate, and methyl 10-hydroxystearate, in which the molecular ion is observed, illustrating the utility of the field ionization source in finding the molecular weight of volatile compounds which fragment easily in the electron impact source.

Electron impact and field ionization mass spectrometers have limited utility when dealing with compounds that are of extremely low volatility or that are subject to thermal degradation. Recently a new and promixing technique, field desorption mass spectrometry (FDMS), has been introduced by Beckey (31) and is being used to study these types of compounds. FDMS is similar to field ionization mass spectrometry in that the electron tunneling process produces the positive ions. The difference in technique is that the sample is deposited on carbon micro needles. The carbon micro needles serve as an anode which enhances and the electric field, and as a sample reservoir so that the sample can be brought to within the few angstroms required to produce ionization (32)

In the field desorption spectra of a triglyceride, the molecular ion generally is the base peak in the spectrum. The M+1 peak also makes a prominent appearance in the spectrum. Only one fragmentation occurs, and peaks result from the loss of RCO_2H from the protonated molecular ion. With the development of the field desorption technique, the qualitative and quantitative identification of triglycerides is apparently possible (33).

PHOSPHOLIPIDS

Electron impact mass spectrometry has found some use in phospholipid analysis, but conventional techniques, such as GC-MS and the use of volatile derivatives, do not generally yield molecular ions from the intact underivatized molecule. Work by Horning et al. (34) has shown that volatile trimethylsilyl derivatives of 1,2- and 1,3-diglycerides, derived from phospholipids, can be studied by GLC and mass spectrometry. Several phospholipids including

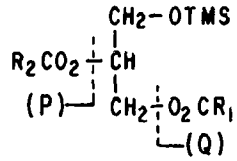

FIG. 14. The generalized fragmentation pattern for a 1,2-diglyceride-3-trimethylsilyl derivative of a phospholipid. (34).

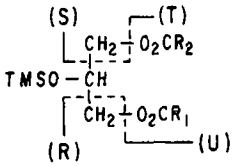

FIG. 15. The generalized fragmentation pattern for a 1,3-diglyceride-2-trimethylsilyl derivative of a phospholipid. (34).

lecithin and cephalin were examined. The mechanism of the reaction involves a thermal cleavage of the alcohol phosphate. The compounds were then studied with electron impact mass spectrometry and characteristic fragmentation patterns permitted the identification of the fatty acids and the location of the fatty acids on the glycerol chain. It is possible to distinguish between the trimethylsilyl ether derivatives of 1,2- and 1,3-diglycerides by mass spectrometry. The 1,2-diglyceride undergoes cleavage at (P) and gives abundant peaks at M-R_2COO and M-R_2COOH (Fig. 14). Cleavage also occurs at (Q) and prominent ions due to M-R_1COO and M-R_1COOH are observed in the mass spectrum. Cleavage at both (P) and (Q) gives rise to a peak at m/e 129, and together with the peaks which appear at M, M-15, and M-90, are characteristic of trimethylsilyl ether derivatives. The long chain fatty acid components give rise to prominent peaks at RCO and RCO + 74.

When compared to the 1,2-isomer, there are significant differences in the mass spectrum of the trimethylsilyl ether derivatives of a 1,3-diglyceride. Cleavage at (R) and (S) is prevalent and results in prominent peaks at M-R_1COOCH_2 and M-R_2COOCH_2 (Fig. 15). Fragmentation at (T) and (U) results in the peaks observed at M-R_1COO, M-R_1COOH, M-R_2COO, and M-R_1COOH.

In general, information about these compounds are garnered from electron impact mass spectrometry only after a considerable expenditure in time and effort. First, a chemical treatment is required to derivatize the compounds, then inferences must be drawn from the spectra

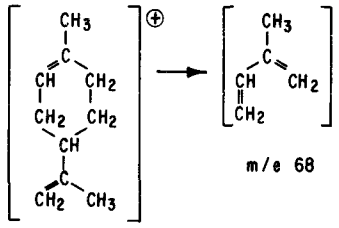

FIG. 16. The Retro-Diels-Alder fragmentation of limonene.

of the resultant reaction products as to the nature of the original compound.

Field desorption studies by Wood et al (35) have demonstrated that this technique provides a way to determine the molecular weight of intact phospholipids. In the field desorption spectrum of phosphatidyl choline, the base peak occurs at M+1, with an abundant peak also occurring for M. Another cluster of peaks corresponds to the loss of choline phosphate, and in general may be useful for establishing the alcohol-phosphate present in the molecule. Peaks corresponding to ion-molecule interactions appear at M-15 and M+15. This mechanism apparently arises from the transfer of a methyl group from the ammonium nitrogen to the oxygen of the phosphodiester. This fact was elucidated through the use of the deuterated quaternary salt. The phospholipids found in biological sources always contain several molecular species, and it is of importance to examine the possibility that field desorption mass spectrometry can be used to analyze such mixtures. Wood and Lau (36) have reported that a mixture of phospholipids, which included dimyristoyl, dipalmitoyl and distearoyl compounds, gave molecular ion peaks which were easily identified. This evidence offers much promise for the determination of the phospholipid composition of biological fluids. It appears that field desorption may be applicable for the analysis of other compounds of limited stability, such as sphingomyelins. One present application of this technique is the identification of intact molecular species present in phosphatidyl choline (lecithin) extracts of amniotic fluid, which may provide insight into the process of fetal lung maturation. The present methods for lecithin analysis leave much to be desired, and should the field desorption method fulfill its potential, there are numerous similar biomedical problems in which the knowledge of the composition of phospholipids or other complex molecules would be of great importance.

TERPENES AND STEROIDS

The use of mass spectrometric techniques in the investigation of terpenes and steroids is commonplace, and the literature that has been published in this area is considerable. Space limitations permit the discussion of only selected examples. An extensive review of the literature on the mass spectra of terpenes has been compliled by Enzel et al. (37). Some of the general features of the mass spectra of terpenes are exemplified by considering the fragmentation patterns of monoterpenes. In general, mass spectrometry can provide the molecular weight in terms of a molecular ion, and suggest the functional groups present in the molecule, but has limitations and other chemical and instrumental techniques must be employed to determine structural and positional isomers, stereochemistry, etc. The fragmentation pattern and peak intensities of acyclic monoterpene hydrocarbons depend on the molecular stability and are thus related to the position of the double bonds. Allylic cleavage is an important fragmentation pathway in this series of terpenes. Fragmentation is also influenced by oxygenated groups, often giving rise to more complex spectra than the corresponding hydrocarbons. Molecular ions of alcohols are generally of low relative abundance. In the cases where the molecular ion is absent, the

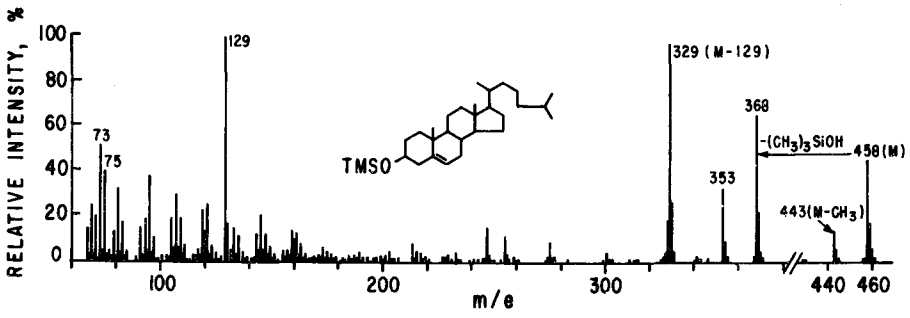

FIG. 17. The mass spectrum of the trimethylsilyl ester of cholesterol (41).

presence of a hydroxyl group may be demonstrated by the presence of M-15 and M-18 peaks. The abundant ions derived from the spectra of monoterpene alcohols and esters are due to hydrocarbon fragments, and prominent peaks are observed at m/e 136, 121, 93, and 69. The characteristic peak at m/e 69 is typical of acyclic monoterpenoids and is due to allylic cleavage of the terminal isopentenyl group.

A prominent M-43 ion is characteristic of most cyclic monoterpenoid spectra. In the hydrocarbons, this peak has a high relative intensity and is often the base peak in the spectrum. The stability in the ring system of the doubly unsaturated hydrocarbons in this series is indicated by prominent peaks at m/e 136, 121, and 93 while only weak ions appear in the lower mass region. A significant exception to this is limonene, which gives a base ion at m/e 68, apparently due to a Retro-Diels-Alder type fragmentation (Fig. 16).

The following work was done in cooperation with Dr. S.F. Osman, Dr. E.B. Kalan, and Dr. S.R. Thomas. An example of the application of GC-MS in the investigation of terpenes involved the structure identification of a unique bicyclic sesquiterpene, which was a major stress metabolite produced by potato tubers *(Solanum tuberosum)* innoculated with the late-blight fungus *Phytophthora infestans.* Using the combined Varian 1700 gas chromatograph interfaced to a CEC 21-492 mass spectrometer, the reduced compound was compared to that of a compound of known structure, verifying its vetispirane carbon skeleton. High resolution mass spectrometry in addition to other techniques aided in the confirmation of the structure as Spirovetiva-1(10),11-dien-2-one (38).

The mass spectra of steroids have been thoroughly reviewed by Budzikiewicz (39,40). One of the most widely used techniques in the investigation of sterols is the use of trimethylsilyl ether derivatives. In general, trimethylsilyl ether derivatives of alcohols are used for purification purposes due to their greatly increased volatility which enables separation by gas chromatography and structure identification by mass spectrometry. The mass spectrum of cholesteryl trimethylsilyl ether illustrates the general appearance of steroidal trimethylsilyl ether spectra (Fig. 17). In the mass spectrum of cholesteryl trimethylsilyl ether, a characteristic feature is a molecular ion of high relative intensity. The base peak in the spectrum appears at m/e 129 and is a usual feature of trimethylsilyl ether compounds. This base peak has been shown to correspond to a trimethylsilyl propene ion, which contains carbon atoms C_1, C_2, and C_3. Another prominent peak in this spectrum is the M-129 ion which results from the expulsion of the neutral trimethylsilyl propyl radical with charge retention on the hydrocarbon skeleton (41). The FDMS of sterols in many cases provides an abundant molecular ion as the only peak in the spectrum. In some cases, the M-18 peak due to the loss of water is present in addition to the molecular ion (4,42,43).

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Studies on the Hydrogen Belts of Membranes: I. Diester, Diether, and Dialkyl Phosphatidylcholines and Polyoxyethylene Glycerides in Monolayers with Cholesterol

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ABSTRACT

The hydrogen belts of membranes are defined as the regions consisting of hydrogen bond acceptors, i.e., the C=O groups of glycero- and sphingolipids, and hydrogen bond donors, i.e., cholesterol-OH, sphingolipid-OH, proteins, and water. Lipid-lipid hydrogen bonding in these belts has been suggested. The connection of such hypothetical bonding with the condensation effect, i.e., the apparent reduction of surface area occupied by phospholipids in mixed monolayers with cholesterol, has been tested with lipids possessing and lacking C=O groups: diester, diether, and dialkyl phosphatidylcholine, and analogous polyoxyethylene diglycerides. Condensation by cholesterol was observed for all lipids. Consequently, the hypothetical lipid-C=O-cholesterol hydrogen bonding is not a prerequisite for the condensation effect.

INTRODUCTION

The regions that separate the hydrophobid core from the outer, polar zones of a membrane have so far received little attention. These regions we call hydrogen belts (1) because they consist of hydrogen bond acceptors (C=O groups of glycero- and sphingolipids) and hydrogen bond donors (cholesterol-OH, sphingolipid-OH, water, perhaps proteins). The balance of these groups in the hydrogen belts is very probably of great biological importance. This is suggested by the difference in belt compositions between manufacturing and isolating membranes of mammals: membranes that manufacture protein (endoplasmic reticulum) or energy (mitochondria) are rich in lipid C=O and very poor in lipid OH groups; on the other hand, the hydrogen belts of the isolating plasma membrane, and especially the myelin membrane, are very rich in hydroxyls. We have suggested (1) that hydroxyl groups close membranes by lipid-lipid hydrogen bonding and that proteins and cholesterol compete for bonding to the ester C=O groups of phospholipids.

Cholesterol plasticizes as well as rigidifies phospholipid bilayers (2). Phospholipid monolayers are condensed by cholesterol, that is, the apparent surface area occupied by a phospholipid molecule is reduced when cholesterol is added (3,4). This effect reflects the intercalation of cholesterol and fatty acid chains; it is maximal with cholesterol and those other sterols that have a flat ring system, a side chain, and a β-oriented 3-hydroxyl (5). Cholesterol and such sterols also reduce the permeability of phospholipid bilayers for non-electrolytes (6) and cations (7). Steric and energetic arguments have led us to suggest (1) that the cholesterol-OH may be hydrogen-bonded to phospholipid C=O, and that such bonding might be a condition for the condensation effect. This hypothesis could be tested with polar lipids lacking the C=O groups, in which the possibility of C=O-cholesterol hydrogen bonding is eliminated. De Kruyff et al. (8) have already investigated monolayers of some relevant model lipids with cholesterol and reached the conclusion that neither the nature of the polar head group nor that of the hydrogen belt groups are responsible for the effect; however, a figure in this study (Ref. 8, Fig. 3) shows that the condensation effect was considerably smaller for a 1-ester-2-ether-phosphatidylcholine than for a diester phosphatidylcholine. To investigate the problem further, we have synthesized a phosphatidylcholine (diester-PC) and its diether and dialkyl analogs, and also some analogs in which the zwitterionic phosphorylcholine is replaced by a polyoxyethylene group. We have then determined the force-area curves, at room temperature, of monolayers of these lipids alone and together with cholesterol.

MATERIALS AND METHODS

All synthetic lipids have been listed with their full names in Table I.

Phospholipids

Phosphatidylcholine (diester-PC), Table I, 1,

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TABLE I

Abbreviations and Structures of Lipids Synthesized

		Structure
1	Diester-PC	1-0-hexadecanoyl-2-0-(*cis*-9'-octadecenoyl)-*sn*-glycero-3-phosphocholine[a]
2	Diether-PC	1-0-hexadecyl-2-0-(*cis*-9'-octadecenyl)-*sn*-glycero-3-phosphocholine[b]
3	Dialkyl-PC A	2,2-di-(9,10-methyleneoctadecyl)ethoxy-1-phosphocholine
4	Dialkyl-PC B	2,2-di(*cis*-9-octadecenyl)ethoxy-1-phosphocholine
5	Diester glyceride A	1,2-0-di(9,10-methyleneoctadecanoyl)-3-0-(2-(2-methoxyethoxy)ethyl)-*rac*-glycerol
6	Diether glyceride A	1,2-0-di(9,10-methyleneoctadecyl)-3-0-(2-(2-methoxyethoxy)ethyl)-*rac*-glycerol
7	Diester glyceride B	1,2-0-di(*cis*-9-octadecenoyl)-3-0-(2-(2-(2-methoxyethoxy)ethoxy)ethyl)-*rac*-glycerol

[a]Position 1 contained 32% stearic acid, 2% other homologs.

[b]Position 1 contained 18% stearyl residue, 12% other homologs.

was prepared by modification of egg lecithin rather than by total synthesis in order to make its fatty acid chain composition similar to that of the available diether-PC. Egg lecithin (9) was deacylated in position 2 with phospholipase 2 (10) and reacylated with oleic acid anhydride (11). The lipid contained 48 mole % oleic acid (18:1), 35 mole % palmitic (16:0), 16 mole % stearic (18:0), and 2 mole % other fatty acids (14:0, 15:0, 16:1, 17:0, 18:2). It was, therefore, mainly 1-palmitoyl-2-oleoyl-PC (nearly 70%) with an admixture of 1-stearoyl-2-oleoyl-PC (about 30%).

Diether-PC (Table I, 2) was prepared from commercial chimyl alcohol (Sigma, St. Louis, MO) which contained 70 mole % 1-palmityl (16:0)-*sn*-glycerol, 18 mole% stearyl analog, and 15% other homologs. It was tritylated in position 3 (12), an oleyl group was introduced in 2 with oleyl bromide and KOH in toluene (12); the product was purified by chromatography and detritylated with 90% acetic acid (13), followed by alkaline hydrolysis of the diether-acetate, to yield 1-alkyl-2-oleyl-*sn*-glycerol. This was converted to diether-PC with 2-bromoethyl dichlorophosphate and trimethylamine (14). Mol wt assuming 75% C_{16}, 25% C_{18} chains in position 1: 757.1, phosphorus calculated, 4.1%, found 4.1%. This lipid contained, according to synthesis, 70 mole % 1-0-palmityl-2-0-oleyl-*sn*-glycerol-3-phosphorylcholine, 18 mole % 1-0-stearyl-, and the rest closely related homologs. The product was pure as judged by thin layer chromatography (TLC), as were all other lipids used in our experiments.

The synthesis of dialkyl-PC B, (Table I, 4) started with a repeated malonic ester condensation of oleyl (18:1) bromide (15). The disubstituted malonic ester was saponified, decarboxylated by heating in vacuum at 180 C, esterified (methanol-H_2SO_4), and reduced with $LiAlH_4$ to the alcohol which was converted as above to dialkyl-PC B, 2,2-dioleylethoxy-1-phosphorylcholine. Mol wt, $C_{43}H_{88}O_4PN \cdot H_2O$, 730.1; P, 4.2%, found 4.2%.

Dialkyl-PC A (Table I, 3) contained two aliphatic chains with a cyclopropane ring. The starting material was 9,10-methyleneoctadecanoic methyl ester [from methyl oleate with diiodomethane and zinc (16), which was reduced with $LiAlH_4$ to the alcohol, which was converted to the bromide with CBr_4 and triphenylphosphine (17)].

Polyoxyethylene Diglycerides

Diester glyceride A, Table I, 5, was prepared by acylation (acid chloride, pyridine) of the substituted glycerol *rac*-3-0-(2-(2-methoxyethoxy)ethyl)-glycerol. This had been prepared from methoxyethoxyethanol which was converted, with PBr_3 (ether, room temperature, 20 hr) to methoxyethoxyethyl bromide, bp 51 C/3 mm, which was condensed with 1,2-isopropylideneglycerol (KOH, toluene) (product bp 112 C/3 mm). The isopropylidene group was then removed with 10% aqueous acetic acid, 2 hr at 100 C. The diester glyceride A was purified by high performance liquid chromatography on Porasil® (Waters Associates, Milford, MA) with hexane-1% isopropanol. On silicic acid thin layer chromatography in hexane: ether, 6:4, its retention was similar to that of a diglyceride. Analysis by gas liquid chromatography after transesterifiecation, with methyl oleate as internal standard: 9,10-methylenestearate, calculated as acid, for the monoester 62.75%; for the diester: 79.9%; found: 79.3%.

Diether glyceride A, Table I, 6, the diether analog of diester glyceride A, was synthesized from *rac*-3-0-(2-(2-methoxyethoxy)ethyl) glycerol and 9,10-methylenestearyl bromide (KOH, toluene). Separation from a slower running product (monoether) was achieved by chromatography on a silicic acid column with hexane:ether, 6:4. The lipid, of which no further analysis is available, was homogeneous by TLC and moved close to diglyceride.

Diester glyceride B, *rac*-3-0-(2-(2-(2-meth-

TABLE II

Area of Phospholipid or Glyceride Molecule in Monolayer at 20 dyn·cm^{-1} and 22 C

		Without cholesterol	With cholesterol, 1:1	% Condensation
1	Diester-PC	72.6 ± 1.5	51.9 ± 1.4	29
2	Diether-PC	75.0 ± 1.0	51.7 ± 0.2	31
3	Dialkyl-PC A[a]	74.4 ± 1.0	59.9 ± 0.4	19.5
4	Dialkyl-PC B[b]	81.3 ± 1.1	61.3 ± 0.6	25
5	Diester glyceride A	70.5 ± 0.6	64.1 ± 2.0	9
6	Diether glyceride A	74.5 ± 1.3	65.5 ± 1.2	12
7	Diester glyceride B	77.9 ± 0.6	74.9 ± 0.2	4

[a]A, di-9,10-methylenestearyl lipids.

[b]B, dioleyl lipids.

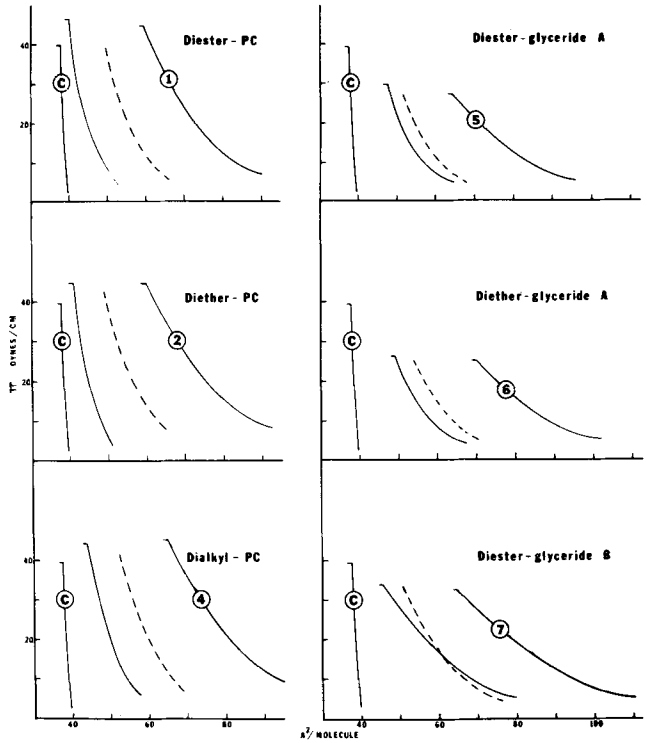

FIG. 1. Force-area curves of lipid monolayers at 22 C. To the right in each graph, with numbers referring to the compounds of Table I, the polar lipid. C is cholesterol. The dashed curve would be expected for an equimolar mixture without condensation. The solid unmarked line is the actually measured force-area curve for an equimolar mixture of polar lipid and cholesterol.

oxyethoxy)ethoxy)ethyl)-glycerol-1,2-dioleate, Table I, 7, is similar to diester glyceride A but contains oleic acid and one additional oxyethylene link. 2-(2-(2-Methoxyethoxy)ethoxy)-ethanol (Olin Chemicals, Brandenburg, KY) was converted with PBr_3 to the bromide; bp 76 C/1 mm; condensated with 1,2-isopropylidene glycerol, product bp 128 C/1 mm; removal of the isopropylidene group with 10% acetic acid; acylation with oleoyl chloride in benzene-pyridine. "Diester glyceride B", mol wt 767.2, calc., C:72.02%; H:11.30%; found, C:72.16%; H:11.07%. In TLC, the lipid behaved like a diglyceride.

Cholesterol was of specially purified grade (Supelco, Inc., Bellefonte, PA) or analytic reagent recrystallized three times from ethanol; no difference was noted between the preparations.

Monolayer Experiments

Lipid monolayers on water were prepared in

a Teflon trough, 30 x 15 x 0.5 cm, with a Teflon slide bar connected to a controllable mechanical drive. Surface tension of the monolayer was continuously monitored with a platinum blade suspended from a No. FTA-10-1 Transducer connected to a No. 8805-P Preamplifier (Hewlett Packard Inc., Paramus, NJ). The signal was continuously recorded. Monolayers were made by gently placing drops of the lipid in hexane (1 mM) on the water surface from a microsyringe. One hundred nmoles of cholesterol, 50 nmoles of lipid, or an equimolar mixture of cholesterol and lipid, 100 or 50 nmoles total, was used for each run, which lasted 12 min. The force-area curves shown have been averaged from four to five measurements taken at room temperature, 22 C. The standard errors for the areas were around ± 1.0 Å^2 (Table II).

RESULTS

Force-Area Curves of the Phospholipids and Condensation by Cholesterol

The force-area characteristics of monolayers of diester-PC, alone and with cholesterol (Fig. 1, 1), agree with those determined by de Kruyff et al. (8) for the same compound. The strong condensation effect is indicated by the distance between the experimental curve (in the middle) and the theoretical (dashed) curve for the mixed lipids. Table II gives surface areas per molecule for diester-PC, as well as the other lipids, at a surface pressure of 20 dyn·cm^{-1}, the apparent area after condensation, calculated assuming cholesterol to be incompressible, and the apparent condensation in percent.

The force-area curve for diether-PC B (Fig. 1, 2) is indistinguishable from that of the diester-PC within the limits of accuracy. Paltauf et al. (18) obtained a similar result with dipalmityl-PC. The condensation effect with cholesterol is of exactly the same magnitude as for diester-PC (Table II, 2).

The dialkyl-PC B monolayer, with the lipid having two oleyl chains, is slightly more expanded (Fig. 1, 4; Table II, 4). Cholesterol again causes condensation, although the area of the condensed phospholipid remains somewhat larger than for diester- and diether-PC. Dialkyl-PC A (Table II, 3; no figure shown), with cyclopropane fatty alkyl chains, has the same surface area as diester- and diether-PC and is also condensed but to a somewhat lesser extent.

Polyoxyethylene Glycerides

The glycerides A containing a methoxyethoxyethoxy polar group (Fig. 1, Table II; 5 and 6) yielded force-area curves similar to those of the phospholipids, but the monolayer collapsed at lower surface pressure. Both lipids were condensed by cholesterol, though only moderately.

The diester glyceride B substituted with a methoxyethoxyethoxyethoxy group (Fig. 1, Table II; 7) formed a somewhat more stable monolayer, with the expected force-area characteristics. Cholesterol caused considerable condensation at high pressure only; at 17 dyn·cm^{-1}, condensation was no longer noted; and at lower pressure the film with cholesterol appeared actually to be expanded.

DISCUSSION

In this study, we set out to determine if the possibility of hydrogen bonding between the carboxyl C=O groups of polar lipids and the hydroxyl of cholesterol was a prerequisite of the monolayer condensation effect. To this purpose, a phosphatidylcholine was compared with analogs lacking its hydrogen bond accepting C=O groups: a diether and a dialkyl phosphatidylcholine. Both compounds displayed the area-condensation effect with cholesterol, the diether-PC to exactly the same degree as diester-PC, the dialkyl-PC somewhat less. It should appear, then, that phospholipid-cholesterol hydrogen bonding, if it exists, is not responsible for the condensation effect. Strictly, however, the results only show that phospholipid-C=O-cholesterol bonding is not required, since the ether oxygen of the diether lipid might also be a potential hydrogen bond acceptor. [The possibility of phosphate=O-cholesterol bonding in the case of the dialkyl-PC will be discussed in the following paper (19)]. However, the ether oxygen is a weaker hydrogen bond acceptor than the C=O group, and molecular models show that it would be less freely accessible to the cholesterol-OH proton. Since the condensation effect for the ether lipid is, nevertheless, exactly equal to that of the diester-PC, it is unlikely that the hypothetical lipid-lipid hydrogen bonding is of critical importance for the effect. The experiment by de Kruyff et al. (8) showing a reduced condensation effect for a 1-ester-2-ether-PC, as compared to diester-PC, does not quite conform to our results; perhaps an ester-ether lipid may behave differently from both diester and diether lipid, though this does not seem likely. In any case, the authors concluded from the condensation effect that did exist, although reduced, that this result provided no evidence for cholesterol-phospho-

lipid bonding in the hydrogen belt. From our results, we arrive at the same conclusion.

The fact that a modification of the hydrogen belt, as performed in our experiments, does not influence the condensation of phospholipid monolayers by cholesterol does not, of course, prove that such a modification will be insignificant in all other respects; nor does it disprove the existence of phospholipid-cholesterol hydrogen bonding. In the following paper (19), we report that an effect of such a modification appears as a change of the activation energy of bilayer permeation.

The polyoxyethylene glycerides were synthesized to serve as membrane lipid analogs containing hydrophilic head groups which, in contrast to those of natural lipids, contained neither electrical charges nor hydrogen bond donors, and only weak hydrogen bond acceptors (the ether oxygens). These lipids formed monolayers somewhat less stable than those of phospholipids (Fig. 1: 5, 6, 7), and we have not been able to prepare liposomes from them. The cyclopropane, long chain lipids were employed in order to eliminate the danger of oxidation and to allow a catalytic hydrogenation step during the synthesis, though this procedure was, eventually, not used. Van Deenen had reported (20) that cyclopropane groups, just as double bonds, conferred fluidity on membranes. As shown by the force-area curves, all lipids were, in fact, in a liquid-expanded state at room temperature.

Both diester and diether glyceride A are condensed by cholesterol (Fig. 1: 5 and 6); again, it is clear that glyceride-C=O-cholesterol hydrogen bonding, if it exists, cannot be indispensible for condensation. The condensation effect is appreciably smaller for the glycerides than for the phospholipids. This might be interpreted as evidence for cholesterol-OH-phosphate=O interaction in the case of phospholipids; however, there are strong theoretical arguments against this possibility (1), as well as counterevidence from nuclear magnetic resonance (NMR) experiments (21), and de Kruyff et al. (8) found that condensation by cholesterol of a glycolipid (which contains neither phosphate nor double-bonded oxygen in the head group) equalled that of a phospholipid. The reason for the smallness of the effect in the polyoxyethylene lipids is probably the low polarity of these compounds, which might at best bind three water molecules to their head group as opposed to the 10-11 tightly bound by phosphorylcholine (22). The polyoxyethylene head groups may, perhaps, be partially inserted into the hydrophobic region of the monolayer and fill the interstices that would receive the cholesterol. Diester glyceride B, with a longer hydrophilic head group, and greater monolayer stability, shows appreciable condensation by cholesterol at high surface pressure, perhaps because the head group is now squeezed out into the aqueous phase. At low surface pressure, addition of cholesterol paradoxically appears to extend the area occupied by the glyceride. It appears that the cholesterol molecules must occupy more than their usual 38.5 $Å^2$ as a result, perhaps, of tilting from their usual vertical position because the aqueous subphase is shielded by polyoxyethylene residues and the monolayer becomes depolarized.

ACKNOWLEDGMENTS

We thank Dr. H.L. Rosano for instructing us how to assemble the surface tension measuring equipment, and Mrs. P.C. Schmidt and Mr. W.D. Bear for expert technical assistance. This study was supported by research grant GM21875 from the National Institute of General Medical Sciences, U.S. Public Health Service.

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Studies on the Hydrogen Belts of Membranes: II. Non-electrolyte Permeability of Liposomes of Diester, Diether, and Dialkyl Phosphatidylcholine and Cholesterol

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ABSTRACT

We have postulated the existence of lipid-lipid and protein-lipid hydrogen bonding in the hydrogen belts of membranes, i.e., the regions of hydrogen bond acceptors (carbonyl oxygens of esters and amides) and hydrogen bond donors (hydroxyls of cholesterol, sphingosine, proteins, water). To assess the possible effects of modifications of the hydrogen belts on membrane permeability, we prepared a diester phosphatidylcholine and two analogs lacking carbonyl oxygens, a diether and a dialkyl phosphatidylcholine, care being taken to synthesize lipids of identical efficient hydrophobic chain length. Relative permeation rates for glycerol and urea were determined by osmotic swelling of liposomes containing the phospholipids alone or with an equimolar quantity of cholesterol, with 4 mole % of dioleylphosphate added. The permeation rates of both solutes were similar for all three lipids, with Arrhenius activation energies ΔE^* around 16 kcal/mole. Cholesterol reduced the permeability of all three membranes. The activation energy ΔE^* of permeation did not change for diester and dialkyl phosphatidylcholine with cholesterol, but was lower by about 5 kcal/mole for the diether lipid with cholesterol. This corresponds to a reduction in the entropy of activation $\Delta\Delta S^* \sim -16$ cal/mole/degree. We interpret the results as supporting the hypothesis of interaction between cholesterol hydroxyl and phospholipid carbonyl.

INTRODUCTION

Between their hydrophobic core and their hydrophilic polar zones, lipid bilayers and biological membranes contain two layers ["hydrogen belts" (1)] consisting of hydrogen bond acceptors (the carbonyl oxygens of lipid ester and amide bonds) and hydrogen bond donors (hydroxyls of cholesterol, sphingosine, proteins, water). The balance of the donor and acceptor groups contributed by the lipids differs from membrane to membrane and is undoubtedly of great biological significance (2). We have suggested that lipids can bind to lipids, especially cholesterol to phospholipids, by hydrogen bonding, and that such bonding might control the permeability and the protein content of the membrane. The membrane-closing faculty of cholesterol has been amply documented (3-6); it has been found that it depends on the 3-hydroxyl group in β-configuration (7). Steric and energetic arguments have been advanced to show that cholesterol-OH-phospholipid-C=O bonding is possible, even probable (1). In pure phospholipid bilayers, the lipid C=O groups must be presumed to be hydrated (1); spectroscopic evidence for such hydration has, in fact, recently been presented (8).

In the following study, we have tried to assess the influence of the hypothetical cholesterol-phospholipid hydrogen bond on the permeation of bilayers by two small nonelectrolytes, glycerol and urea. We have substituted for the diester phospholipid, phosphatidylcholine, a diether and a dialkyl analog in which the possibility of a C=O···HO bond is eliminated. The relative permeabilities were measured by the osmotic swelling of multi-layered liposomes of these phospholipids with and without cholesterol.

MATERIALS AND METHODS

Lipids

The phosphatidylcholine, "diester-PC," was essentially 1-palmitoyl-2-oleoyl-*sn*-glycerol-3-phosphorylcholine (70%) with 30% of the 1-stearoyl homolog, prepared as described in the preceding paper (9).

Diether phosphatidylcholine, "diether-PC," containing 70% 1-0-palmityl-2-0-oleyl-*sn*-glycerol-3-phosphorylcholine, 18% 1-0-stearyl, and 16% other homologs, was prepared as described (9).

Dialkyl phosphatidylcholine, "dialkyl-PC,"

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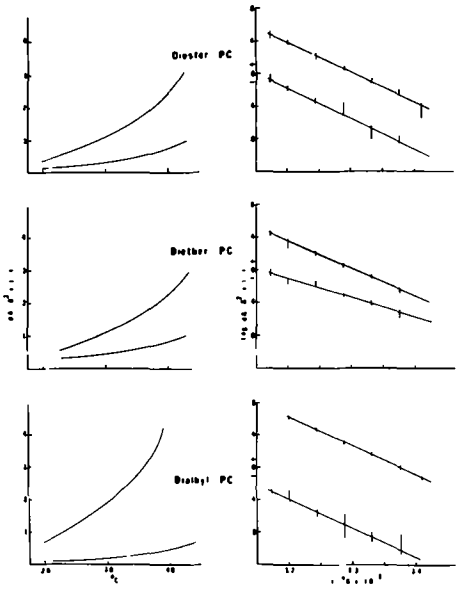

FIG. 1. Three determinations of relative rates, as measured by $(dA/A^2)sec^{-1}$, of permeation of glycerol through phospholipid liposomes. All lipids with 4 mole % dioleyl phosphate added. At left: upper curve in each graph, the phospholipid; lower curve, phospholipid with equimolar cholesterol. At right: the corresponding Arrhenius plots; bars give range of data.

was synthesized as follows: myristyl ($C_{14:0}$) bromide was condensed with malonic ester; the recrystallized product was condensed (10) with palmitoleyl bromide, 1-bromohexadec-*cis*9-ene, which had been prepared from palmitoleic acid ester (Nu-Chek Prep, Elysian, MN) via the alcohol ($LiAlH_4$ reduction) with CBr_4 and triphenylphosphine (11). The disubstituted malonic ester was saponified (KOH in ethanol), monodecarboxylated (2 hr in vacuum at 180 C), esterified (methanol-H_2SO_4), and reduced to the alcohol ($LiAlH_4$), which was then converted to the phosphatidylcholine analog by condensation with 2-bromoethyl dichlorophosphate followed by trimethylamine (12). The product was purified by preparative thin layer chromatography (TLC) on silicic acid with chloroform:methanol:water, 64:34:5. *Rac*-2-tetradecyl-2-hexadec-*cis*9-enyl-ethoxy-1-phosphorylcholine, $C_{37}H_{76}O_4PN \cdot H_2O$, mol wt 648.0; P, calc. 4.78%; found, 4.9%.

Dioleyl phosphate was synthesized from oleyl alcohol (70% oleyl, the rest palmityl and other homologs) and phosphorus oxychloride, and isolated by preparative TLC (silicic acid, chloroform:methanol:water 32:8:1); phosphorus, calc., 3.18%; found, 3.26%. Cholesterol was an ultrapure preparation (Supelco, Bellefonte, PA) or a three times recrystallized product of analytical grade.

Liposome Swelling

Published procedures were followed (13). Liposomes were prepared from hexane solutions containing 40 μmoles of lecithin and 1.6 μmoles of dioleyl phosphate [added to assure trapping of sufficient water between the bilayers (13)], or 20 μmoles lecithin, 20 μmoles cholesterol, and 1.6 μmoles of dioleyl phosphate. Solutions were evaporated to dryness in a 25 ml round-bottom flask in water vacuum and then oil pump vacuum at 50 C, with frequent flushing with dry nitrogen. Two glass beads were then added to the flask, and the lipid was dispersed in 4 ml of 0.15 M KCl, first with agitation by hand and then by 1 min vortexing. The final concentration of total lipid was 10.4 mM. Liposomes were always prepared in the afternoon and left standing overnight at room temperature.

Swelling experiments were performed with the help of a Gilford 2400 spectrophotometer equipped with a Gilford 2445 Spectro-Stir (Gilford Instrument Laboratories, Inc., Oberlin, OH). The temperature was controlled with a circulating water bath. A volume of 1.5 ml (degassed) 0.3 M aqueous glycerol or urea solution (isotonic with the entrapped 0.15 M KCl) was placed into a cuvette in the Spectro-Stir cuvette holder, and allowed to reach the experimental temperature. A microsyringe was then inserted through a hole in the cuvette chamber cover, and 40 μl of liposome suspension were injected into the solution. The stir disc inside the cuvette accomplished mixing within 1 sec. The recorder, at a chart speed of 30 cm/min, registered the initial increase in absorbance at 450 mμ (1-2 sec) and the following decrease resulting from liposome swelling. The decrease was linear between 2-5 sec after injection and was measured during this period at 5-7 different temperatures from 23 C–42 C. At least five determinations were made at each temperature.

The change in absorbance divided by the square of the initial absorbance, per second, $(dA/A^2)(1/dt)$, is inversely proportional to the volume change of the liposomes and the permeability coefficient (13). Thus, the values of $(dA/A^2)(1/dt)$, as presented in Figure 1, allow a comparison of the permeability coefficients although the absolute values are not being determined.

RESULTS

All three phospholipids (with 4% dioleyl phosphate added) formed liposomes. Table I shows the actual optical density values meas-

ured for 0.40 μmoles of handshaken liposomes in 1.5 ml isotonic KCl. Since the absorptivity is directly proportional to the number of vesicles, and inversely proportional to their size, the data indicate that diester-PC and diether-PC form multilayer liposomes of very similar size distribution and that their size is changed in the same way when cholesterol is added. Dialkyl-PC seems to form liposomes which are larger and fewer in number, but with cholesterol it again forms liposomes very similar to those of the other two lipids. These phenomena have not yet been further studied; neither have transition temperatures and enthalpies been measured, though the appearance of the temperature-permeability curves (Fig. 1) makes it clear that all lipids were in their liquid-crystalline state at room temperature.

Figure 1 presents the results of one set of experiments each for the three lipids, without and with cholesterol, for glycerol at different temperatures, and the corresponding Arrhenius plots used for the determination of the activation energies ΔE^*. The curves obtained for permeation by urea (not shown) are very similar. Table II lists the relative permeabilities for both glycerol and urea at 37 C; the data were obtained from four to five determinations. Values of ΔE^* are listed in Table III. The rates of glycerol permeability are very similar for diester- and diether-PC, and roughly 50% higher for dialkyl-PC (Fig. 1; Table II); the same is true for urea permeation (Table II). Cholesterol closes the membranes of all three lipids, most efficiently that of the dialkyl-PC. The activation energy for glycerol penetration of diester-PC bilayers, 17 kcal/mole, agrees more closely with that found by de Gier et al. (13), 19 kcal/mole, than with that reported by Cohen (5), 11 kcal/mole. In agreement with de Gier et al., we find no change in ΔE^* when cholesterol is added to diester-PC. Cohen (5) reported an increase in ΔE^*.

For the diether-PC, the ΔE^* of permeation by glycerol and by urea (Table III) is similar to that for the ester-PC; but, in contrast, in the presence of 1 mole of cholesterol, ΔE^* is lowered for both permeants by about 5 kcal/mole. In addition to the experiments reported in Table III, where the phospholipid:cholesterol

TABLE I

Optical Density (OD) of Dispersions of Liposomes[a]

	OD	With cholesterol
Diester-PC	0.53 ± .06	.46 ± .03
Diether-PC	0.57 ± .02	.44 ± .05
Dialkyl-PC	0.37 ± .05	.41 ± .03

[a]Hand shaken, containing 0.40 μmoles phospholipid, or 0.20 μmoles phospholipid and 0.20 μmoles cholesterol, all with 4% dioleyl phosphate (0.016 μmoles), at 22 C in 1.5 ml of 0.15 m aqueous KCl, at 450 mμ, 1 cm pathlength.

TABLE II

Relative Permeabilities, as $(dA/A^2)sec^{-1}$, of Liposomes, Without and With Cholesterol (1:1 mole), All With 4 mole % Dioleyl Phosphate, at 37 C

Lipid	Permeant	$(dA/A^2)sec^{-1}$ Without cholesterol	$(dA/A^2)sec^{-1}$ With cholesterol
Diester-PC	glycerol	1.92 ± 0.08	0.64 ± 0.07
	urea	1.40 ± 0.09	0.61 ± 0.06
Diether-PC	glycerol	1.85 ± 0.10	0.71 ± 0.07
	urea	1.25 ± 0.13	0.63 ± 0.03
Dialkyl-PC	glycerol	2.86 ± 0.50	0.36 ± 0.02
	urea	2.30 ± 0.40	0.39 ± 0.08

TABLE III

Activation Energies ΔE^* of Permeation of Glycerol and Urea through Lipid Bilayers[a]

Permeant	Glycerol		Urea	
Lipid	–	With cholesterol	–	With cholesterol
Diester-PC	16.6 ± 1.8	16.8 ± 1.4	15.9 ± 0.8	16.2 ± 1.7
Diether-PC	15.4 ± 1.3	10.1 ± 0.9	15.4 ± 0.7	9.9 ± 1.1
Dialkyl-PC	15.7 ± 1.4	15.6 ± 2.2	15.0 ± 1.4	15.8 ± 1.3

[a]Temperature, 23-42 C. Liposomes contained 96 mole % phospholipid and 4 mole % dioleyl phosphate; or 48 mole % phospholipid, 48 mole % cholesterol, and 4 mole % dioleyl phosphate.

ratio was 1:1, two runs were performed on liposomes of a ratio 1:0.7. These also yielded an average ΔE^* of 10.0 kcal/mole for the permeation of diether-PC by glycerol.

For the dialkyl-PC, the activation energies resembled those for the diester-PC, and they were not changed when cholesterol had been added at a 1:1 molar ratio (Table III).

DISCUSSION

Effective Hydrophobic Chain Length

The permeability of bilayers is influenced by the chain length and the degree of unsaturation of the hydrophobic phospholipid chains; a reduction by two carbons or an introduction of one double bond per molecule approximately doubles the rate of permeation (14). Therefore, if the influence of the hydrogen belt composition on permeability is to be studied, the lipids tested must be equal not only in the structure of the polar group but also in their hydrophobicity. According to Tanford (15), the carbon carrying oxygen and its neighbor do not contribute to the hydrophobic effect (i.e., the negative free energy of transfer of lipid from aqueous to hydrocarbon phase), and a double bond has an effect somewhat larger than that of the loss of one CH_2 group; we choose to set one double bond equal to the loss of two CH_2 groups (2). The effective hydrophobic chain length of our diester-PC, then, is 28 (exactly, because of the admixture of 30% stearoyl homolog, 28.6). This number is also obtained for our diether-PC (more exactly, around 28.4) and dialkyl-PC. Since an increase of 2 in the effective hydrophobic chain length causes an increase of around 100% in permeability, and the effect is additive in lipid mixtures (14), the nonhomogeneity of diester-PC and diether-PC in their hydrophobic parts could cause a difference of not more than 10% in permeability between them, or 20-30% between them and the dialkyl-PC. Furthermore, the activation energies of permeation, on which the following arguments rest, are virtually independent of the fatty acid composition (16).

Permeability for Glycerol and Urea

The relative rates of permeation for the small solutes, as expressed by $(dA/A^2)dt^{-1}$, are approximately equal for diester- and diether-PC (Fig. 1; Table II). For dialkyl-PC (without cholesterol), $(dA/A^2)dt^{-1}$ is about 50% larger (Table II); this may indicate a slightly different molecular packing in the bilayer, as it is also indicated by the different average size of the liposomes (Table I). The activation energies of permeation are identical for all three lipids (Table III). It is generally agreed that penetration of the membrane is preceded by the total dehydration of the solute and that the activation energy, ΔE^*, relates to this process (5,16,17). It follows, then, that the presence or absence of the phospholipid C=O or ether oxygen groups has no bearing on solute dehydration, and that the carbonyl oxygen does not play the role of a catalyst in permeation.

The addition of cholesterol to the diester-PC depresses the rate of permeation but has no influence on ΔE^*; this is in agreement with a report from de Gier et al. (16). If we assume the existence of C=O-cholesterol hydrogen bonding, this result would be expected since the C=O groups, as we just stated, are not directly involved in solute dehydration. The dialkyl-PC membrane is also closed by cholesterol (Fig. 1), without there being a change in activation energy (Table II).

The bilayers containing diether-PC behave in a different manner. The phospholipid alone displays a permeability very similar to that of the diester (Fig. 1) with a ΔE^* of the same magnitude. Cholesterol again closes the membrane (Table II). However, the activation energy of permeation for the mixed diether-PC-cholestero bilayer is reduced by about 5 kcal/mole (Table III). A similar phenomenon was observed by Johnson and Bangham (18) when they measured potassium ion penetration in phosphatidylcholine liposomes: the activation enthalpy dropped by $\Delta\Delta H^* = -5$ kcal/mole when both valinomycine and an anesthetic were incorporated in the membrane. [ΔH^* is slightly lower, by 0.6 kcal/mole, than ΔE^* (17)]. We can follow their interpretation of the effect. Since the permeability coefficient is an exponential function of the free energy of activation (17), ΔG^*, and the permeabilities of the three lipid bilayers with cholesterol differ only insignificantly from each other (Table II), it follows, all other conditions being equal, that $\Delta\Delta G^*$ (the difference of free energies of activation between the three kinds of liposomes) is nearly zero. The difference in the enthalpy of activation $\Delta\Delta H^* = -5$ kcal/mole must, therefore, reflect a difference in the entropy of activation, ΔS^*. From $\Delta G^* = \Delta H^* - T\Delta S^*$ (17), we calculate $\Delta\Delta S^*$ as -16 cal/mole/degree. It follows that the entropy of the ground state must be higher by 16 cal/mole/degree in the case of the diether-PC-cholesterol bilayer.

A Molecular Model

We have proposed (1) that the ester-C=O groups of phospholipid bilayers accept hydrogen bonds from water and that the hydroxyl

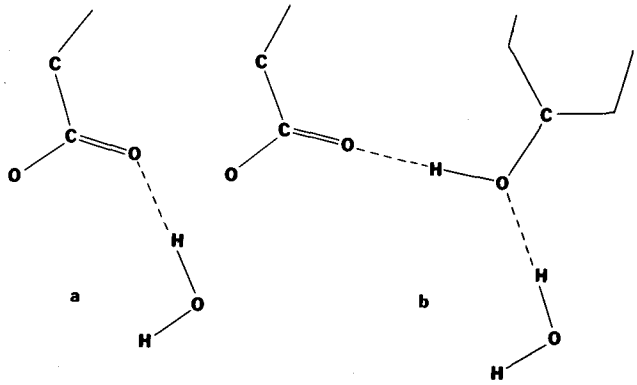

FIG. 2. Hypothetical alignment of phospholipid ester groups and hydrogenbonded water (a) and realignment in the presence of cholesterol (b).

group of added cholesterol bonds to the C=O, displaces the water but accepts a hydrogen bond of its own from water at the back of the hydroxyl=O. Such rearrangement (Fig. 2) would not alter the general longitudinal orientation (ie., perpendicular to the membrane (1)] of the O-H-bonds of the water partaking in the hydrogen belts. We can fit our results into this model. The attainment of the transition state of solute dehydration is accompanied by a negative change of entropy of activation, i.e., by increased ordering of the system. (A positive change, which possibility we do not want to deny, would change the sign of our argument but not is content). Water and solute being the only reactants involved, a transitory ordering of water molecules can be expected to take place. This ordering may be facilitated by the preexisting orderly alignment of water in the hydrogen belts of the phospholipid bilayers. To account for the identical entropy content of the ground state of permeation for all three lipids, the same alignment shown for one water molecule and ester group in Fig. 2, a, may also pertain to water-diether-PC bonding (with the ether oxygen possibly as acceptor) and to water-dialkyl-PC bonding (with P=O as acceptor, replacing C=O). Insertion of cholesterol, which would not cause disalignment of water in the diester-PC hydrogen belt (Fig. 2, b), would also not cause disalignment for dialkyl-PC if the cholesterol would hydrogen bond to phsophate oxygen. There are strong arguments (1) and evidence (19,20) against cholesterol-phosphate bonding in diester phospholipid layers; in the dialkyl-PC, however, the phosphate has moved much nearer to the hydrophobic part of the molecule, and therefore to the hydrophobic core of the membrane containing cholesterol, and bonding between polar head group and cholesterol-OH may well be possible. This hypothesis may be open to experimental verification (19). In the diether-PC-cholesterol membrane, however, lipid-lipid hydrogen bonding has become difficult or impossible. The cholesterol-OH can neither approach the phosphate, because the hydrophilic character of the glycerol ether system forces the cholesterol to retreat into the membrane; nor can it easily bond to the ether oxygens, because they are weaker hydrogen bond acceptors than ester-C=O and are sterically not so readily accessible; space-filling molecular models make this apparent. As a result, the cholesterol O-H bond is no longer mostly latitudinally oriented [i.e., parallel to the membrane (1)], the alignment of the water molecules is upset, and the entropy content of the system increased.

The finding that our diether lipid-cholesterol system and the anesthetic-valinomycin-phosphatidylcholine system of Johnson and Bangham (18) show a similarly increased ΔS^* of the ground state of permeation lends weight to our suggestion (1) that many drugs may act on membranes by disarranging the hydrogen belts.

The molecular model of Figure 2 must be regarded as tentative and certainly imcomplete; for instance, several additional water molecules can be fitted between the hydrogen belt and the polar head groups of the lipids, and these must also somehow affect the dehydration of the solute, since in the case of glycerol, for example, no fewer than six hydrogen bonds have to be broken. Nevertheless, we hope that the model, if too simple, is not entirely incorrect. At any event, our results show that there is an energetical difference between cholesterol-ester lipid and cholesterol-ether lipid membranes, therefore a difference in intermolecular structure, and since the systems differ only in the

presence and absence of ester carbonyl groups, an interaction between these groups and cholesterol is implicated. In a recent comparison of a diester-PC and a diether-PC, in aqueous dispersions alone and in mixture with cholesterol, Schwarz et al. (21) arrived at the same conclusion on the basis of differences found in bilayer structure parameters.

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SHORT COMMUNICATIONS

Analytical ^{13}C NMR: Detection, Quantitation, and Positional Analysis of Butyrate in Butter Oil

ABSTRACT

The amount of butyrate contained in a complex mixture of butter oil triglycerides was 10.3 mole % as determined by natural abundance ^{13}C Fourier transform pulse nuclear magnetic resonance (NMR) spectroscopy. This NMR technique also demonstrated the primary isomeric positioning (>97%) of the butyrl group without the need for altering or fractionating the fat mixture.

INTRODUCTION

The analysis of triglyceride structure has been extensively researched (1). Enzymatic techniques, employing pancreatic lipase, have been a major tool in this work and have resulted in the elucidation of the positional distribution of fatty acids in triglycerides in a large number of natural fats (1), including milk fat (2-5). Nevertheless, when triglycerides, such as those in milk fats, are the substrates under study, the utility of the enzymatic probes has been questioned (6-8). Thus, investigators have taken great pains to develop experimental conditions and alternate techniques in their attempts to satisfactorily interpret data emanating from pancreatic lipase studies of milk fat triglycerides (9-14).

The problems associated with the studies of milk fat triglycerides are obviated when the glycerides are examined directly without prior chemical or enzymatic modification. Nuclear magnetic resonance (NMR) is such a probe and, indeed, studies utilizing proton NMR, in combination with achiral and chiral chemical shift reagents, have been employed to verify the primary versus secondary positioning of fatty acids in synthetic triglycerides which contain saturated chains in combination with either unsaturated (15-17) or branched (18) chain fatty acids and to substantiate the stereospecific positioning of buryric acid in a buryrate rich fraction of hydrogenated milk fat (19).

Our studies with Carbon-13-NMR (C-NMR) confirm the primary positioning of butyric acid in unaltered milk fat triglycerides, and we show that this technique can be easily adapted for the quantitative analysis of butyryl esters by the direct examination of milk fat and other butyryl glycerides.

MATERIALS AND METHODS

Triglycerides

Tributyrin (BBB) was purchased from Eastman Kodak (Rochester, NY) and purified by elution with petroleum ether (30-60 C) through neutral alumina (20). Glyceryl-*rac*-1,2-dipalmitate-3-butyrate (*rac*-PPB), glyceryl-*rac*-1,3-dipalmitate-2-butyrate (*rac*-PBP), and glyceryl-*rac*-1,2-dibutyrate-3-oleate (*rac*-BBO) were synthesized and purified in the laboratory of Dr. R.G. Jensen (University of Connecticut, Storrs, CT) according to established procedures (6,21). Optically clear butter oil was obtained by churning fresh, pasteurized cream, melting the butter at 42 C, and storing overnight at 4 C. The butter was remelted at 63 C, and the buttermilk was removed. The fat phase was centrifuged at 5000 rpm, the oil was removed and filtered through glass wool.

^{13}C-NMR

^{13}C-NMR spectra were taken on a Bruker WH-90 Fourier transform NMR spectrometer, operating at 22.63 MHz with proton noise decoupling, and with a Varian CFT-20 operating at 20.0 MHz. The alkyl region of the spectrum was obtained at a 2000 Hz sweep width and displayed at a sweep width of 500 Hz. Pulses of 90° were used with 1000-2000 accumulations. The spectra were obtained in 0.5-1 M solutions of $CDCl_3$ with all chemical shifts relative to internal tetramethylsilane (TMS). The accuracy of the reported δ-values are within ± 0.02 ppm. Repetition rates of 10 sec were utilized to assure quantitative peak area relationships (22). Measured NOE values for all carbon resonances utilized for quantitative composition evaluations were 2.9 ± 0.15.

RESULTS AND DISCUSSION

During our ^{13}C-NMR studies of the *cis/trans* ratio of various fats and oils (22), we observed an unusual aliphatic chemical shift pattern which was exclusive to butter fat. Based on cal-

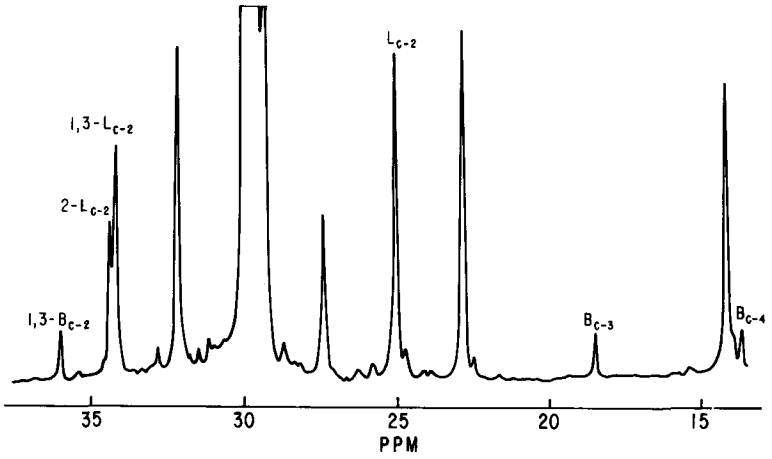

FIG. 1. 22.63 MHz ^{13}C spectrum of butter oil (0.5 g in 1.3 ml of $CDCl_3$). Sweep width of displayed spectrum is 500 Hz. The letter (B) identifies shifts due to butyrate and (L) those due to long chain species.

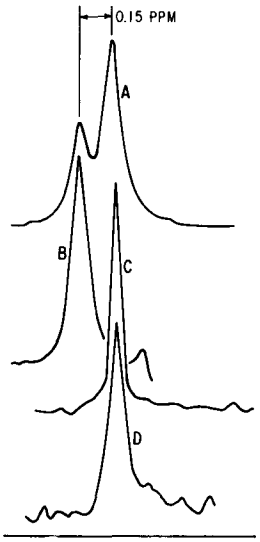

FIG. 2. Plot expansion of butyrate C-2 resonance in (A) BBB, (B) *rac*-PBP, (C) *rac*-PPB, and (D) natural butter oil.

TABLE I

^{13}C Resonance Positions of C-2 in Butyryl and Long Chain Mixed Triglycerides[a]

	δC-2 butyryl		δC-2 long chain	
Triglycerides	1,3	2	1,3	2
Butter oil	35.94	---	34.07	34.24
BBO	35.96	36.14	34.09	---
PPB	35.98	---	34.14	34.30
PBP	---	36.14	34.11	---
BBB	35.96	36.12	---	---

[a]All chemical shifts are reported relative to internal (TMS) in $CDCl_3$.

culated shielding constants (23), the single isolated carbon resonances observed at δ13.63, 18.40, and 35.94 were assigned to carbons 4, 3, and 2 of a butyl ester chain, respectively. The ^{13}C spectrum of butter oil and the position of the three butyrate carbon resonances and other relevant shifts are shown in Figure 1.

By relating the area of the C-3 resonance at δ18.40 to the C-3 resonance area centered at δ24.97, representing all other fatty acid chains, we obtained directly the mole % butyrate content of the unmodified mixture as follows:

$$\text{mole \%, butyrate} = 100 \times \frac{\text{C-3 of butyrate}}{\text{C-3 of butyrate + C-3 of long chains}}$$

Analysis of a freshly prepared butter oil by this procedure showed 10.3 ± 0.3 mole % butyrate content while the value obtained by gas liquid chromatography (GLC) analysis of the derived butyrate (24) was 9.8 mole % ± 0.3%.

On close inspection of the C-2 resonance associated with the butyrate moieties, we observed that, unlike the long chain counterparts, this shift was characterized by a single peak position, (δ35.94). In the long chain esters, however, two C-2 resonances at δ34.07 (area 2) and δ34.24 (area 1) were observed, reflecting esterification at the 1,3- and 2-positions of glycerol, respectively (25). From these observations, it seemed reasonable to conclude that butyrate was restricted primarily to a single isomeric position of glycerol, i.e., 1,3- or 2- in butter. To verify this conclusion and make the correct positional assignment. we examined the ^{13}C spectra of several model compounds including those whose structures corresponded to

the possible compositions we were studying in the natural system. Comparison of the C-2 resonance position as observed in butter oil, *rac*-PPB, *rac*-PBP, *rac*-BBO, and BBB directly confirmed the predominance of butyrate in the primary positions of the butter oil triglycerides (Fig. 2). The limits of detection of any secondary butyryl esters of glycerol in butter were established through a study of mixtures of known composition to be no less than 3% of all butyrate containing triglycerides. In addition to butyrate, the isomeric composition of acetate and propionate (23) triglycerides can also be ascertained in the presence of longer chain esters. However, hexanoate and those of higher molecular weight cannot, due to their common C-2 resonance position. The C-2 shift positions of various butyrate containing triglycerides are listed in Table I.

In previous high field (220 MHz) proton NMR studies (19), stereospecificity, i.e., *sn*-3 positioning of butyrate chains, was ascertained through pseudo contact shift methods following fat fractionation and sample doping with synthetic optical isomers. However, these results were not entirely unambiguous since the pure *sn*-2 isomer was not examined.

Our present report confirms the findings of previous workers (2-5,19) and removes any ambiguities concerning the isomeric positioning of butyric acid in milk fat triglycerides, since no fractionation or alteration of the triglyceride occurred. It also points the way to the further exploitation of ^{13}C-NMR for direct routine quantitative analysis of a variety of unaltered lipid derived materials. A more detailed report concerning the quantitative aspects of this procedure as it pertains to the limitations of detection of butyrate, hexanoate, and free fatty acids will be the subject of a future publication.

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Chain Length Specificity in the Utilization of Long Chain Alcohols for Ether Lipid Biosynthesis in Rat Brain

ABSTRACT

A mixture of *cis*-9-[1-^{14}C]octadecenol and [1-^{14}C]docosanol was injected into the brains of 19-day-old rats, and incorporation of radioactivity into brain lipids was determined after 3, 12, and 24 hr. Both alcohols were metabolized by the brain but at different rates; each was oxidized to the corresponding fatty acid, but oleic acid was more readily incorporated into polar lipids. Substantial amounts of radioactivity were incorporated into 18:1 alkyl and alk-1-enyl moieties of the ethanolamine phosphoglycerides and into 18:1 alkyl moieties of the choline phosphoglycerides. Even after the disappearance of the 18:1 alcohol from the substrate mixture (12 hr), the 22:0 alcohol was not used to any measurable extent for alkyl and alk-1-enyl glycerol formation.

INTRODUCTION

We have recently identified saturated alcohols ranging in chain length from 16 to 24 carbon atoms and the monounsaturated alcohol octadecenol in developing rat brain (1). Since the alkyl and alk-1-enyl groups of the ether lipids of rat brain and of most other mammalian tissues have a remarkably simple composition and consist mainly of saturated and monounsaturated moieties of 16 and 18 carbon atoms (2), we postulated a chain length specificity for the utilization of fatty alcohols in ether lipid biosynthesis (1). In the present paper, we demonstrate such a chain length specificity for the biosynthesis of ether bonds in rat brain in vivo.

EXPERIMENTAL PROCEDURES

cis-9-[1-^{14}C]octadecenol (59 mCi/mmol) and [1-^{14}C]docosanol (52 mCi/mmol) were prepared by $LiAlH_4$ reduction of the corresponding fatty acid methyl esters (Dhom Products, Ltd.) and were purified by thin layer chromatography (TLC) to a radiopurity of better than 99%. An aqueous emulsion of about equal amounts (55 and 45% of dpm) of the labeled alcohols containing bile salts (10 mg/ml, Sodium Choleate, Sigma Chemical Co.) was injected intracerebrally to 19-day-old rats (3) (ARS Sprague Dawley, Madison, WI). The animals, in groups of 4 or 5, were killed after 3-24 hr, the brains were removed immediately, pooled, and homogenized in chloroform: methanol (2:1, v/v). The lipids were extracted (4) and stored in chloroform at -20 C. Isolation of phospholipid classes, degradation by methanolysis, acidic hydrolysis and hydrogenolysis with $LiAlH_4$, preparation of isopropylidene derivatives and alkyl acetates, and fractionation by TLC and gas liquid chromatography (GLC) were done as described previously (3,5). Argentation TLC was done on Silica Gel G (Merck) containing 12.5% (w/w) silver nitrate. The plates were prewashed with ethyl acetate before being activated at 110 C for 1 hr.

RESULTS AND DISCUSSION

In order to establish patterns of metabolism

TABLE I

Incorporation of Radioactivity from Long Chain Alcohols into Rat Brain Lipids

Substrate administered per brain:	
[1-^{14}C]octadecenol	3.12×10^5 dpm (2.38 nmol)
[1-^{14}C]docosanol	2.55×10^5 dpm (1.98 nmol)

Radioactivity (dpm x 10^{-4}) recovered per brain:

	Time (hr)		
	3	12	24
Total lipids	44.99	33.89	29.45
Alcohols	28.16	16.21	10.36
Other nonpolar lipids	1.44	1.49	2.12
Total phospholipids	15.23	16.35	16.69
Ethanolamine phosphoglycerides	6.30	6.56	5.29
Choline phosphoglycerides	7.93	8.87	7.35

of fatty alcohols of different chain length, a mixture of *cis*-9-[1-^{14}C]octadecenol and [1-^{14}C]docosanol was administered to 19-day-old rats by intracerebral injection. Previous work had shown (5) that [1-^{14}C]octadecenol is efficiently incorporated into alkyl and alk-1-enyl moieties of the ethanolamine phosphoglycerides, into alkyl moieties of choline phosphoglycerides (6) and, after oxidation to oleic acid, into the acyl moieties of brain phospholipids (5). The use of a saturated and a monounsaturated alcohol as substrate was also of technical advantage because both GLC and argentation TLC, methods which complement each other, could be employed in the analysis of the metabolic products.

Data on the administration of the substrates and on the recovery and distribution of radioactivity in the major brain lipid fractions are summarized in Table I.

As expected, substantial amounts of radioactivity were incorporated into ethanolamine and choline phosphoglycerides. Most of the radioactivity present in the neutral lipids remained with the long chain alcohols; small amounts were also associated with other fractions corresponding in their migration rates to wax esters and triglycerides.

An aliquot of the total lipids was subjected to acidic methanolysis, and the fatty acid methyl esters and dimethyl acetals were isolated by TLC. From another aliquot of the total lipids, the long chain alcohols were isolated and were acetylated for analysis by GLC and argentation TLC after adding unlabeled alcohols as carriers. Methyl esters and dimethyl acetals were separated into saturated and monounsaturated fractions by argentation TLC. As summarized in Table II, the dimethyl acetals showed virtually no radioactivity in the saturated fraction, whereas the saturated methyl esters contained substantial amounts of radioactivity, proportionately increasing with time.

These data were in good agreement with the results obtained by GLC (Table II). Radioactivity was more rapidly incorporated into 18:1 than into 22:0 acyl groups. Only minor amounts, increasing with time to less than 10%, were associated with 16:0 acyl groups, an indication that not much of the radioactivity in long chain acyl moieties was due to degradation and resynthesis. The results obtained by argentation TLC of the dimethyl acetals were fully confirmed by GLC of the aldehydes derived from the alk-1-enyl moieties of the ethanolamine phosphoglycerides by acid cleavage.

Acidic methanolysis of the total *neutral* lipids yielded labeled fatty acid methyl esters (Table II) of which 22:0 contained relatively

TABLE II

Radioactivity (%) in Aliphatic Moieties of Total Lipids, Neutral Lipids, and Remaining Substrate 3, 12, and 24 hr After Administration of [1-^{14}C]Octadecenol and [1-^{14}C]Docosanol

Time (hr)	Radioactivity (%) in monounsaturated fractions[a]			Radioactivity (%) in individual structures[b]											
				18:1			22:0			16:0			Others		
	3	12	24	3	12	24	3	12	24	3	12	24	3	12	24
Total acyl groups	84.8	78.8	67.2	88.8	74.2	67.8	7.6	14.4	17.5	–	6.8	6.9	3.6	4.6	7.6
Acyl groups in neutral lipids	Not determined			72.8	46.5	29.1	27.2	41.5	50.2	–	3.1	5.4	–	8.9	15.3
Alk-1-enyl groups	100.0	100.0	100.0	100.0	100.0	100.0	–	–	–	–	–	–	–	–	–
Unmetabolized alcohols	24.5	2.7	–	25.6	2.2	–	74.3	97.8	100.0	–	–	–	–	–	–

[a]Determined by argentation thin layer chromatography.
[b]Determined by gas liquid chromatography.

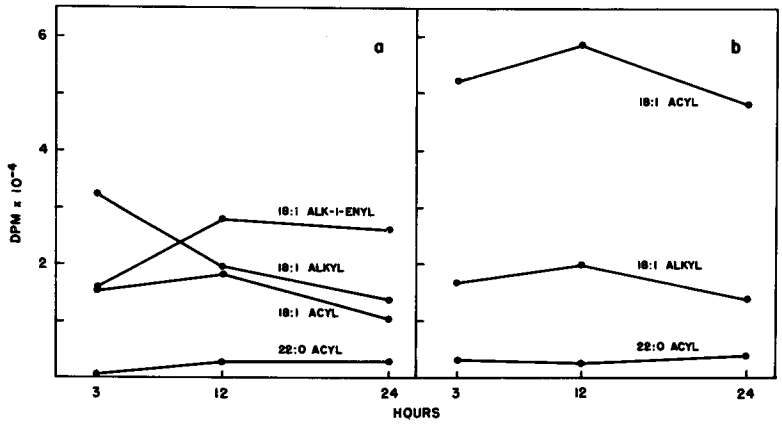

FIG. 1. Radioactivity per brain in various long chain moieties of ethanolamine (a) and choline (b) phosphoglycerides 3, 12, and 24 hr after intracerebral injection of *cis*-9[1-^{14}C]octadecenol and [1-^{14}C]docosanol.

high amounts of radioactivity which, in the 24 hr group, exceeded that found in 18:1.

From the data in Table II, it is clear that both alcohols were metabolized by the brain but at different rates. Each was oxidized to the corresponding fatty acid but oleic acid was more readily incorporated into polar lipids. Incorporation of both fatty acids into neutral lipids proceeded more slowly but showed increasing proportions of docosanoic acid, obviously because of the availability of docosanol over a longer period of time.

Since docosanol apparently was not incorporated into the alk-1-enyl moieties of rat brain ether lipids, it was important to determine the biosynthetic step at which this chain length discrimination occurred. As intracerebrally administered alcohols are incorporated into the alkyl moieties of both ethanolamine and choline phosphoglycerides, these lipid classes were isolated and subjected to acidic methanolysis and hydrogenolysis with lithium aluminum hydride. Analysis of the fatty acid methyl esters, dimethyl acetals, and isopropylidene derivatives of the alkyl glycerols gave the results shown in Figure 1.

Both phospholipid classes exhibited the expected labeling pattern in their 18:1 moieties. In the ethanolamine phosphoglycerides, a decrease in radioactive alkyl moieties corresponded to an increase in alk-1-enyl moieties as expected from a precursor-product relationship of these structures, whereas the choline phosphoglycerides did not contain measurable amounts of labeled alk-1-enyl moieties. Both lipid classes also contained relatively high amounts of labeled 18:1 acyl moieites. Smaller amounts of radioactivity were present in 22:0 acyl groups and none in 22:0 alkyl or alk-1-enyl groups. It follows that, even after the disappearance of the 18:1 alcohol from the substrate mixture (12 hr), the 22:0 alcohol was not used to any measurable extent for alkyl and alk-1-enyl glycerol formation in the developing brain.

In previous work from this laboratory, we have established that alkyl glycerolipid synthesis in developing rat brain proceeds with a variety of fatty alcohol analogues (5-10). However, in all these experiments, the total chain length of the substrates did not exceed the equivalent of a straight chain of 20 carbon atoms. It is reasonable to assume that alcohols of more than 20 carbon atoms cannot serve as substrates in the alkyl-acyl exchange reaction (11,12) leading to *O*-alkyl dihydroxyacetone phosphate, the first intermediate in ether lipid biosynthesis.

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A New Rotifer-Based Assay for Tocopherol

ABSTRACT

The body-wall-outgrowth response of the rotifer *Asplanchna sieboldi* to tocopherol has been developed into a new bioassay which can estimate tocopherol titres more than 10^6 times more dilute than the detection limits of previously reported bioassays, and which is more sensitive and permits greater qualitative discrimination than any fluorometric assay. This rotifer bioassay may prove especially useful in determining biological activities of tocopherol compounds and in estimating total tocopherol activity in biological material of very limited size or tocopherol content. It may also be used to analyze and describe the effects of environmental, genetic, and pharmacological factors on the body-wall-outgrowth responses of *Asplanchna* to given doses of tocopherol in terms of regression equations or equivalent changes in inducing tocopherol concentrations.

INTRODUCTION

After exposure to low concentrations of emulsified tocopherol (vitamin E), the large, predatory rotifer *Asplanchna sieboldi* produces offspring with variably-sized body-wall outgrowths (1); such individuals have been classified into five morphotypes (2). This morphological response is consistent (3), specific for tocopherol (1,3,4), independent of lipid contamination (3-6), and sensitive to as little as 10^{-13} and sometimes 10^{-14} M *d*-α-tocopherol (3,7). Previous studies (1,2,4) proposed that the *Asplanchna* response be used in a quantitative bioassay for tocopherols. Recently, bioassay techniques (8) have been applied to the response, and a bioassay has been developed (9). The present report briefly describes this bioassay, discusses its applicability, and compares it with other analytical techniques – two rat bioassays and a fluorometric assay.

MATERIALS AND METHODS

A. sieboldi is reared on the ciliate *Paramecium aurelia* in the dark at a temperature between 23 and 28 C. Various methods for the cultivation of this and closely-related species of *Asplanchna* have been published (10-13). Full descriptions of all media, food organisms, equipment, culture techniques, and bioassay procedures with calculations are available (9).

In brief, the bioassay is performed by placing newborn rotifers in 35 x 10 mm plastic Petri dishes containing 5 ml of medium with *Paramecium*, Penicillin G, and graded concentrations of emulsified *d*-α-tocopherol, other active compounds such as *d*-β-tocopherol, or unknowns. Tocopherol influences the development of the embryos of these rotifers in a concentration-dependent fashion, the sizes of the lateral, posterior, and posterodorsal body-wall outgrowths varying directly with the tocopherol dosage. The shapes of individuals classified as morphs 1 (no body-wall outgrowths) to 5 (maximal body-wall outgrowths) have been described and illustrated (2,14). Statistical analysis of morph frequencies of offspring produced in dilutions prepared from different compounds permits evaluation of compound similarity. Similarity further permits quantitative estimation of relative potency.

Qualitative discrimination of compounds requires testing for interaction in a 3-way contingency table (preparation x concentration x morph) (15). In the absence of interaction,

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MATERIALS AND METHODS

A. sieboldi is reared on the ciliate *Paramecium aurelia* in the dark at a temperature between 23 and 28 C. Various methods for the cultivation of this and closely-related species of *Asplanchna* have been published (10-13). Full descriptions of all media, food organisms, equipment, culture techniques, and bioassay procedures with calculations are available (9).

In brief, the bioassay is performed by placing newborn rotifers in 35 x 10 mm plastic Petri dishes containing 5 ml of medium with *Paramecium*, Penicillin G, and graded concentrations of emulsified *d*-α-tocopherol, other active compounds such as *d*-β-tocopherol, or unknowns. Tocopherol influences the development of the embryos of these rotifers in a concentration-dependent fashion, the sizes of the lateral, posterior, and posterodorsal body-wall outgrowths varying directly with the tocopherol dosage. The shapes of individuals classified as morphs 1 (no body-wall outgrowths) to 5 (maximal body-wall outgrowths) have been described and illustrated (2,14). Statistical analysis of morph frequencies of offspring produced in dilutions prepared from different compounds permits evaluation of compound similarity. Similarity further permits quantitative estimation of relative potency.

Qualitative discrimination of compounds requires testing for interaction in a 3-way contingency table (preparation x concentration x morph) (15). In the absence of interaction,

TABLE I

Morphotype Frequencies of Offspring from *Asplanchna sieboldi* (Clone 10C6) Exposed to Different Concentrations of *d*-α-Tocopherol Preparations

	Log relative tocopherol concentration												
	Standard							Test 1			Test 2		
Morph	-10	-9	-8	-7	-6	-5	-4	-9	-7	-5	-9	-7	-5
1	126	133	141	105	40	10	16	141	87	12	137	97	13
2	22	34	39	67	15	1	2	26	82	3	26	78	3
3				3	80	11	1			19			15
4					21	19	3			23			17
5						66	67			52			71

TABLE II

ANOVA[a] with Linear Regression for Illustrative Bioassay of *d*-α-Tocopherol Preparations (see Table I)

Source of variation	DF	MS	F	$P(H_0)$
Preparations	2	2.07	0.16	0.851
Linear regression	1	9271.88	732.6	<<0.001
Nonparallelism	2	1.97	0.16	0.858
Nonlinearity	5	63.03	4.98	0.013
Error	11	12.66		

[a]ANOVA = analysis of variance.

TABLE III

Summary Comparison of Tocopherol Assays

	Assay			
Assay characteristics[a]	Rat resorption-gestation	Rat haemolysis	Spectrofluorometry	Rotifer
Specificity:				
tocopherols vs. tocotrienols	No	No	No[f]	Yes
tocopherols and tocotrienols vs. other vitamin-E-active compounds	No	No	Yes	Yes
among substituted chroman-ring derivatives	No	No	?	Yes
among epimers	No	No	No	Yes
Sensitivity:				
working minimum (M)[b]	10^{-4}	10^{-2}	10^{-8}	10^{-10}
response minimum (M)[c]	10^{-5}	10^{-3}	NA	10^{-14}
Precision:[d]				
λ	[.06][e]	.11	.03	.27
$s_\lambda(\nu)$	[.04(1)]	.01(108)	.003(52)	.06(11)
$\hat{n}(\hat{n}_{max})$	[7(618)]	29(46)	2(2)	166(358)
replication units	rats	rats	samples	pairs of Petri dishes

[a]Abbreviations: ? = not known; NA = not applicable.

[b]Lowest sample titre permitting dilution with acceptable intervals.

[c]Lowest concentration to which animals respond.

[d]Symbols; λ = ratio of square root of error MS to regression coefficient; s_λ = standard error of λ; ν = degrees of freedom for s_λ; $\hat{n}$ = estimated sample for dosage estimation error of 10%; $\hat{n}_{max}$ = upper 95% confidence limit for $\hat{n}$.

[e]Square brackets indicate approximation based on angular transformation of published data.

[f]From Taylor et al. (20).

angular transformation (arc $\sin\sqrt{p}$) applied to the relative frequencies (p) of morph 1 offspring allows analysis of variance (ANOVA) with tests for parallel regressions against dosage (log tocopherol concentration).

In an illustrative bioassay, three preparations of ethanolic *d*-α-tocopherol at 10^{-2} M (labeled the 10^{0} concentration) were serially diluted in tenfold steps with rotifer medium. The first or "standard" preparation was allocated to four replicate culture dishes, each containing ten rotifers, at relative concentrations from 10^{-4} to 10^{-10}. The other two "test" preparations were assayed at only the 10^{-5}, 10^{-7}, and 10^{-9} levels.

RESULTS

Table I shows the morphotypes of the offspring. For the nine comparable treatments (10^{-5}, 10^{-7}, 10^{-9} of each of the three preparations), the interaction G was not significant ($P = 0.650$). Table II shows the ANOVA.

Inverse prediction was as follows. For the first "test" preparation, actually at 1.1×10^{-2} M, the estimate was 1.2×10^{-2} M (lower and upper 95% confidence limits were 1.77×10^{-3} M and 8.48×10^{-2} M). The other "test" was actually at 1.09×10^{-2} M and was estimated as 1.10×10^{-2} M (confidence limits were 1.61×10^{-3} M and 7.71×10^{-2} M).

DISCUSSION

Table III compares the specificity, sensitivity, and precision of the rotifer bio-assay with those of the rat dialuric-acid haemolysis assay (16,17), the rat resorption-gestation assay (17,18), and the spectrofluorometric assay (6); details are presented elsewhere (9).

The *Asplanchna* response to tocopherol is more specific than these other assays; it discriminates among some stereoisomers of tocopherol, among some tocopherols with different chroman-ring substituents, and between tocopherol and tocotrienol (3,4) (Table III). Because of its great specificity, the rotifer bioassay may have advantages over the other bioassays in determining the biological activities of naturally-occurring and synthetic tocopherol compounds, especially if these are available in only small amounts. For example, although preliminary studies indicated that α- and β-tocopherols were similarly active in triggering the rotifer response (1), precise comparisons between these compounds and between *d*- and *dl*-α-tocopherols would be desirable. Also, comparisons of α-tocopherol with analogs having one, two, or three fewer carbons on the side chain or having one or more fluorine atoms substituted for hydrogen atoms on the methyl groups of the benzene ring might be instructive in determining the mechanism of tocopherol action in *Asplanchna*.

The rotifer response permits an assay at least 10^{6} times more sensitive to tocopherol than either rat response and at least 10^{2} times more sensitive than spectrofluorometry (Table III). Because of its great sensitivity, the rotifer bioassay could be especially useful in estimating the tocopherol activity in biological material of limited size or very low tocopherol content. If such material contained a mixture of tocopherols, such as α- and β-tocopherols, the contribution of each compound to the biological activity could not be assessed. In this respect the rotifer bioassay is no worse than the fluorometric assay; in either case chromatographic separation of the tocopherols would have to precede their determination.

Another attribute of the rotifer bioassay is that it is generally unaffected by lipid contamination and thus could probably be used for impure preparations. This bioassay has not yet been used to determine tocopherol activities in preparations from biological material, but the *Asplanchna* response to known doses of *d*-α-tocopherol is not altered by the presence of relatively large amounts of crude, tocopherol-free lipids extracted from yeast (6), grass (3), or the rotifer *Brachionus* (5).

The rotifer bioassay, like the other bioassays, has the shortcoming of being much more time-consuming, inconvenient, and imprecise than the fluorometric assay. However, once arrangements for rotifer culture have been made, an assay can be completed within 4 days – much less time than that required for the rat resorption-gestation assay.

Finally, the rotifer bioassay should prove useful in analyzing the *Asplanchna* body-wall-outgrowth response itself. Clonal, geographic, and temporal variability in responses to tocopherol could be studied rigorously and expressed as regressions of morph 1 frequencies vs. tocopherol concentration or if these regressions were parallel, as inducing tocopherol concentrations. Similarly, environmental factors thought to alter the response to tocopherol, such as population density (19), could be evaluated quantitatively. Also, the bioassay could assess the effects of pharmacological treatments on the body-wall-outgrowth response to tocopherol and, therefore, could assist in analyzing the mechanism by which tocopherol influences the pattern of development.

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Pulmonary Surfactant: Distribution of Lipids, Proteins and Surface Activity in Ultracentrifugation of Rabbit Pulmonary Washing and Derived Fractions

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ABSTRACT

Lavage from normal adult rabbit lung and two known derived fractions, Fraction T and Fraction S, were subjected to either differential ultracentrifugation in 1.090 g/ml KBr or sucrose density gradient ultracentrifugation; the surface activity of the lipid extract of selected fractions was measured. In differential ultracentrifugation, the three starting materials yielded a pellicle containing > 85% of the phospholipid with <1% protein. In sucrose density gradient ultracentrifugation: pulmonary washing, containing about equal weights of phospholipid and protein (60% albumin, 20% sIgA, 10% IgG, 10% minor proteins), produced one single band at density 1.040, containing virtually one single protein, namely >80% of the total sIgA (protein T) and up to 60% of the total phospholipid, whereas all the albumin and IgG were found at very low densities, 1.010 and 1.025, respectively; Fraction T, having nearly equal weights of one single protein, sIgA, and phospholipid, produced two contiguous bands at densities 1.059 and 1.078, totalling >85% of its phospholipid and <25% of its protein, the balance of which was found free of phospholipid at densities 1.020 to 1.050; comprising >80% of the phospholipid and <20% of the protein of pulmonary washing, Fraction S yielded two small bands at densities 1.028 and 1.044 and a major band at d = 1.059. In surface activity measurements: when the total lipid extract of the bands from the sucrose density gradient ultracentrifugation was spread as a film, in spite of similarly high dipalmitoyl lecithin contents (about 70% palmitoyl residue), the lipid of the band of Fraction T and that of the high density band of Fraction S were very active ($\gamma_{min} = 0$); whereas the lipid of the band of pulmonary washing and that of the lowest density band of Fraction S were not active, γ_{min} being 18 dyne/cm and 21 dyne/cm, respectively. This work brings forth three major conclusions. First, under conditions which are used to isolate serum lipoproteins, no lipoprotein was obtained from either of the three surfactant fractions and most of the lipid was found virtually free of protein. Second, the isopicnic equilibrium of a given ultracentrifugation fraction varied with the molecular structure of its constituents and could not be accounted for by the latter's average densities; instead, major roles must be played by particle geometry and their water contents. Third, although the various lipid samples contained the same quantities of palmitoyl residues (70%), the surface activities varied with the physical state of the lipid, method of assay, and some other undefined factors.

INTRODUCTION

Although it is recognized that dipalmitoyl lecithin (DPL) is the essential component of pulmonary surfactant (1-4), there is no evidence as yet for the origin, state, and role of certain major proteins (albumin, IgG, and sIgA) that were found with the lipid in rabbit pulmonary washing and in two derived surfactant fractions: Fraction T, which contains sIgA as the only major protein, and Fraction S, which comprises most of the phospholipid of pulmonary washing and small quantities of albumin, IgG, and sIgA (5,6).

Several surfactant fractions have been derived from pulmonary washing, a surfactant preparation by itself (2). Different procedures yielded highly surface active fractions with little protein from dog (7) and rabbit (2) lung washings. In contrast, the surfactant fraction obtained by King and Clements (8) from dog tracheal lavage contained appreciable amounts of a small MW protein (about 10,000 dalton), which was identified as lung specific and part of the lung surfactant system (8-10). Two other proteins of MW 62,000 and 36,000, found in the pulmonary washings of various species, have also been related to pulmonary surfactant (9,11-13). Another surface active preparation

was isolated from homogenate as well as pulmonary washing of dog lung; this fraction which was erroneously thought to be a specific lipoprotein surfactant, consisted of about 80% lipid and 20% protein (14); the latter had the electrophoretic mobility of protein T (2) or sIgA (6). Recent studies (6,15) have established that protein (14) to be also IgA, which is present in the pulmonary washing of several species (15,16), is not an alveolar protein (17) and cannot therefore be a component of alveolar surfactant. The relative quantities of proteins as well as lipids were made to vary with the methods of lung lavage (18).

Two phenomena are evident from a rapid examination of this background. First is the enormous diversity in the preparative procedures, diversity which must be considered when one is trying to account for the marked differences in yield, composition, and surface activity of the given fractions; this phenomenon was briefly referred to by King and Clements (Ref. 19, p. 724). Second, most of the cited methods considered only a selected, relatively small fraction, either in a band in the density gradient, in a pellicle floating on heavy medium, or in a pellet, whereas most of the phospholipid surfactant was either discarded (13,14) or lost in the many-step procedures, including dialysis (8).

Using differential ultracentrifugation and primarily sucrose density gradient ultracentrifugation, we studied the behavior of rabbit pulmonary washings and Fractions T and S (5,6). Unlike previous reports (8,13,14), we examined the lipid and protein composition of all the aliquots of the density gradient instead of an arbitrarily selected band.

MATERIALS AND METHODS

Pulmonary Washing

This was obtained from freshly excised lungs of New Zealand white rabbits (3 to 4 kg) by the following method. The rabbit was anesthetized with 15 mg/kg pentobarbital and then sacrificed by transection of the abdominal aorta. The trachea was clamped during inhalation, and the lungs were removed en block and put on crushed ice. Saline, at room temperature, 10 ml/g wet lung, was introduced with a syringe via trachea, aspirated, and pushed back gently at the rate of 0.20 cycle/min; at the end of the second cycle, the milky white lavage was collected (60 to 70 ml from 100 ml of starting saline). The crude pulmonary washing was centrifuged at 1,000 x g, 0 C, for 20 min, in order to remove cells (mostly macrophages) and other debris; centrifugation at 750 x g for 10 min (13) yielded identical results.

Fraction T (FxT) and Fraction S (FxS)

The supernatant of the first (low speed) centrifugation, or cell-free PW, was concentrated 20 x by vacuum dialysis at 4 C; 10 ml of concentrated PW, equivalent to 200 ml of original PW, was applied to a Sephadex G-200 column that was equilibrated with 1 mM tris-HCl in 1 M NaCl, containing 1 mM EDTA, pH 7.5. After the usual elution (11), the fraction collected in the void volume (Fraction I) was dialyzed against 0.15 M NaCl and then centrifuged at 49,000 x g for 1 hr at 4 C. The supernatant, referred to as FxT contained most of protein T and a nearly equal weight of phospholipid. The pellet, FxS, contained most of the lipid of dialyzed Fraction I and a mixture of three proteins, serum albumin, γ-globulin, and sIgA (6). The lipid and protein compositions were similar to those already published for both fractions (5,6). The phospholipid/protein ratio varied from 0.5 to 1.5 in FxT and 4 to 8 in FxS.

Protein and Lipid Standards

Fibrinogen and thyroglobulin were obtained from Sigma Chemical Co., St.Louis, MO; lysozyme from Worthington Biochemical Co., Milburn, NJ; and ovalbumin from Schwarz-Mann Laboratories, New York, NY. Rabbit serum albumin and γ-globulin were from Pentex, Kankakee, IL. Lipid standards were purchased from Supelco (Bellefonte, PA). Chromatographically homogeneous methyl esters of 10:0, 12:0, 14:0, 14:1, 16:0, 16:1, 18:0, 18:1, 18:2, and 20:0 fatty acids were products of Applied Science Laboratory, State College, PA.

Aqueous dispersions of lipid, protein, and lipid-protein systems were attained by a 5 min ultrasonic irradiation in ice at 80 watts in a sonifier cell disruptor (Model W 185D, Branson Sonic Power Co., Plainview, Long Island, NY).

Thin Layer Chromatography (TLC) of Lipids

The lipids of given fractions were extracted into chloroform by shaking the aqueous fraction once with 4 vol of chloroform:methanol, 2:1, and a second time with 4 vol of chloroform. The two phases were separated by centrifugation in the cold. The lipids were then fractionated and identified by their migration on TLC plates precoated with Silica Gel G (Analtech, Newark, DE), using chloroform: methanol:conc. ammonia:water, 70:30:2:4, and hexane:ether:acetic acid, 80:30:1, mixtures as developing solvent systems for the separation of phospholipids and neutral lipids (20), respectively. The lipid spots were shown either by staining with I_2 vapor or by charring

after spray with sulfuric acid. The phospholipids were detected by molybdenum trioxide spray (21). Ninhydrin spray (0.2% in 95% ethanol, acidified with glacial HAc) was used to reveal free amino groups on the TLC plates.

The lipid composition was determined by densitometry and gas liquid chromatography against appropriate standards. The phospholipid and neutral lipid spots were scraped off the TLC plates and extracted with chloroform: methanol:ether, 1:1:1, and chloroform: methanol:water, 1:2:0.8, respectively (22). The recovery was better than 95% when tritiated DPL and ^{14}C-palmitic acid were used as markers. The lipid residue was subjected to phosphorus analysis in the case of phospholipids and to methylation and gas liquid chromatography (GLC) for all the lipids, except for cholesterol, which was estimated by densitometry of the charred spot after spraying with 50% H_2SO_4. Quantitation of the lipids by GLC was performed by adaptation of the method described in Kates (22).

Protein and Phosphorus Determinations

Protein concentration was estimated by the method of Lowry et al. (23) using 0.2% SDS as a dispersing agent and bovine serum albumin as a standard. Phosphorus was determined according to a modification of the method of Beveridge and Johnson (24), using perchloric acid for digestion; the phospholipid content was obtained by multiplying the phosphorus content by 25.

Disc Gel Electrophoresis

Polyacrylamide, 7.5%, was used in the presence of 0.2% SDS according to the method of Weber and Osborn (25), except that no reducing agent was used. Proteins of known MW served as standards; the protein bands were stained with Coomassie blue and quantitated densitometrically at 550 nm.

To verify the identity of the proteins that we have always referred to as serum albumin and IgG (5,6), the two proteins were separated by preparative electrophoresis of pulmonary washing on slab gel (7.5% polyacrylamide), using tris-glycine buffer, pH 8.8. The proteins were extracted from the gel with small amounts of 0.1% SDS in 0.05 M ammonium bicarbonate, dialyzed against water, and lyophilized. Of equal aliquots of each protein, 50 μg each, one-half were incubated with 2% β-mercaptoethanol, 3 hr at 37 C and then 2 min at 100 C, using rabbit serum albumin and IgG as standards. Samples of the reduced and nonreduced proteins from pulmonary washing were run side by side in slab gel electrophoresis as before.

Density Gradient Ultracentrifugation

The aqueous sample, 1 to 3 ml, was layered on top of a linear gradient which was prepared in 38.5 ml cellulose nitrate tube (Beckman) by mixing 0.1 M and 1.0 M sucrose (grade I, Sigma) in appropriate gradient maker. A 2 ml 2 M sucrose cushion was used. The gradients were centrifuged in SW 27 rotor at 80,000 x g for 18 hr at 4 C in a Beckman L2-65B preparative ultracentrifuge. Aliquots and/or visible bands were collected from the bottom using a piercing device and a photoelectric volumetric dispensor (Büchler Instruments, Fort Lee, NJ). The gradient was first divided in 16 aliquots, and, after analyses, for convenience the aliquots were combined in pairs to make the eight fractions that are described in tables and figures. The same technique was used to study density gradients in electrolyte solutions between 0.15 M and 1.75 M NaBr or KBr.

Differential Ultracentrifugation

The cell-free pulmonary washing in 0.15 M NaCl (d = 1.006) was made to density 1.090 by addition of solid KBr at 15 C and centrifuged in polycarbonate tubes of 38.5 ml capacity in a fixed angle rotor (Beckman Model 50.1) at 110,000 x g for 24 hr at 15 C in a preparative ultracentrifuge, Beckman Model L2-65B. The lipid pellicle was separated from the infranatant and analyzed for lipids and proteins. The differential ultracentrifugation was carried out at 15 C, since this temperature was used in the isolation of serum lipoproteins (2,26). Dispersions of concentrated Fraction T and Fraction S were diluted to contain 200 μg/ml phospholipid in 0.15 M NaCl, were then sonicated 15 min in ice (above), and finally subjected to ultracentrifugation. Sonication of the samples had no effect on the results. With selected samples, the ones containing unusually large protein contents, we used centrifugations in sequential density steps, i.e., 1.006, 1.063, and 1.210 g/ml KBr (2,26) with the view to ascertain if lipoproteins were present in the pulmonary washing.

Gas Liquid Chromatography (GLC)

GLC was performed in a Barber-Colman (Chicago, IL) apparatus. To the dry lipid sample, 1 ml mixture of methanol:boron trifluoride:benzene, 3.5:3.5:3.0, was added, and methylation was carried out according to Morrison and Smith (27). The fatty acid esters were extracted in hexane, and the resulting solution was injected into the GLC column. The latter, 6 ft long, consisted of B_2-ethylene glycol succinate 15% on anachrom ABS 90/100 mesh P. Temperature of the column was 170 C, and

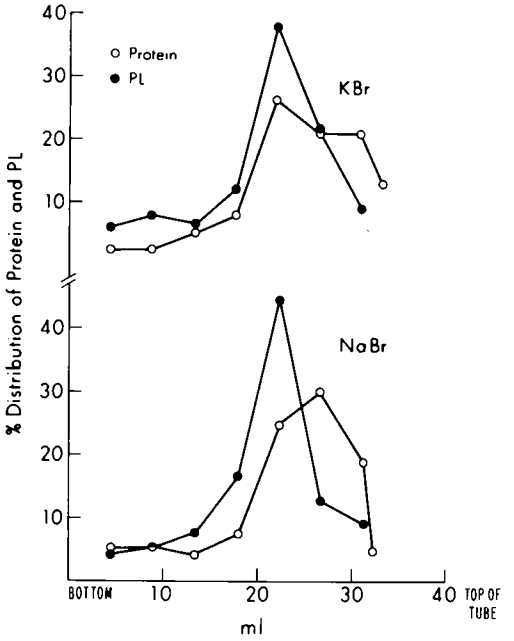

FIG. 1. Percent distribution of phospholipid (PL) (•) and protein (o) in linear NaBr and KBr density gradients of rabbit pulmonary washing.

flow rate of the carrier gas, argon, was 60 ml/min.

Surface Activity

The dynamic surface tension, in continuous cyclic compression and decompression of the surfactant film, was determined by a modified Langmuir-Wilhelmy method according to the procedure described by Clements and Tierney (28). The trough, having 65 cm^2 (100%) useful area, was carved out of a heavy Teflon block; the film was contained by a winding Teflon strip guided by a light Teflon piston, which was connected to the automatic compression-decompression mechanism.

Surface tension was measured by a platinum plate suspended from a Cahn electrobalance. The amplified output of the electromechanical transducer and the area changes between 100% and 20% were fed, respectively, into the Y and X axes of an X-Y recorder, (Esterline Angus, Model XY-8511, Indianapolis, IN). The films were prepared by spreading the given fraction from either aqueous or organic medium on the aqueous hypophase, which consisted of 0.15 M NaCl, at 25 C. The solutions of reagent grade salts were prepared in water that was distilled twice (once over alkaline permanganate) and were foamed in order to remove surface active contaminants (29).

RESULTS

Although each experiment was repeated two to four times, each set of data represents one single experiment; the trends in all similar experiments were consistent.

Lipid and Protein Composition of Rabbit Pulmonary Washing

Since the lipid composition of rabbit pulmonary washing is known (30) and the major lipids are unmistakably identifiable by migration on TLC plates, we did not seek new identification, which is also beyond the scope of this communication. Using the same lipids, we are only comparing the lipid patterns of the various centrifugation fractions.

As already reported by us (5) and others (31), the PL and protein contents of individual rabbit pulmonary washings varied between 100 and 600 μg/ml each. Such variations became smaller when the washings of 6 to 24 rabbits were pooled; then, the phospholipid and protein contents ranged from 250 to 350 μ/ml each, with most of the values between 300 and 350 μg/ml. Although most of the individual lavages had a phospholipid/protein ratio near unity, we have seen preparations in which that ratio was as high as 3.0 and as low as 0.3. However, in all cases, although the quantities of each lipid and protein could vary, there were no new lipids or proteins beside the ones that have always appeared in the lavage of normal rabbit lung, as in the following patterns: phosphatidyl choline $\geqslant$ cholesterol $\geqslant$ phosphatidyl ethanolamine $\cong$ phosphatidyl glycerol $>$ sphingomyelin $\geqslant$ cholesteryl ester $>$ free fatty acid $\geqslant$ phosphatidyl serine; albumin $\gg$ sIgA $>$ IgG. Occasionally we saw PW preparations with protein pattern sIgA $\geqslant$ albumin, especially when the lavage was prepared with ice cold saline in ice cold lung, or after the lung had been perfused with 1 μM ritodrine, a β-mimetic (18).

In line with previous results from gel filtration of normal rabbit pulmonary washing (5), disc gel electrophoresis of the latter showed an average protein distribution of 60% serum albumin, 20% sIgA (protein T), 10% IgG, and 10% minor proteins; among these, invariably appeared a protein of small MW, about 10,000. For simplicity, in the disc gel electrophoresis patterns we do not show the other minor proteins. The unstained albumin-like and IgG-like bands were eluted from the gel; after reduction with β-mercaptoethanol, they yielded the expected products as revealed by gel electrophoresis and immunodiffusion, thus identifying the two proteins as albumin and IgG. The identify of IgA was reported elsewhere (6).

The lipid composition of PW had the following pattern: 70% phospholipid and 30% nonphospholipid or neutral lipid. The latter consisted of cholesterol (50%), cholesteryl ester (30%) and free fatty acid (20%); there were only traces of triglyceride. More than 70% of the phospholipid was lecithin, which, by GLC, had $\geqslant$ 70% palmitoyl residue; the lecithin and other lipids for the GLC analysis were recovered from the TLC plates (see Methods).

Differential Ultracentrifugation

After 24 hr centrifugation in 1.090 g/ml KBr at 110,000 x g, 15 C, no pellet resulted from either the cell-free pulmonary washing, Fraction T or Fraction S. The pellicle contained more than 80% of the phospholipid and virtually no protein, in a phospholipid to protein ratio of about 100/1; the trace amounts of protein in the pellicle consisted of albumin and sIgA, both of which could be nonspecifically entrapped in the lipid particles. All the protein (albumin, IgG and sIgA) was found in the infranatant, as indicated by protein analysis (Lowry) and by densitometry of the electrophoresis gels.

Density Gradient Ultracentrifugation

The results of two types of experiments are presented: one with gradients of NaBr and KBr in Figure 1 and one with gradients of sucrose in Figure 2 and Table I.

Pulmonary washing in NaBr and KBr density gradients (Fig. 1): The density profiles of these two gradients are similar to those of the corresponding sucrose gradients (below). In all the gradients, the phospholipid peaked sharply with the band at density near 1.040. The protein peaked either with the phospholipid, as in KBr, or at a slightly lower density, as in NaBr and in sucrose. In general, the protein was distributed in the low density regions of the gradient as shown also with the sucrose density gradient of pulmonary washing (below).

Sucrose density gradients: A graphic representation of gradient and distribution of proteins in disc gels and lipids on TLC plates is shown in Figure 2 for pulmonary washing and Fraction T. Fraction S was omitted for simplicity, since the protein and lipid patterns were similar to those of pulmonary washings. The percent distribution of phospholipid and protein of the various gradient regions and bands is summarized in Table I for pulmonary washing, Fraction T, and Fraction S.

(a) *Pulmonary washing (Fig. 2, Table I):* A single band appeared at density 1.040, containing most of sIgA (T) and about 33% of the total phospholipid; a considerable quantity of phospholipid centrifuged together with the balance of protein T on the high density side of the visible band, i.e., in tubes 4, 5, and 6. In this particular sample, sIgA represented about 35%, as opposed to an average 20% of the total protein content of pulmonary washing. The abundance of sIgA was confirmed by the amount of this protein in tubes 3, 4, 5, and 6. The protein in tubes 7 and 8 was essentially the low MW protein mentioned above.

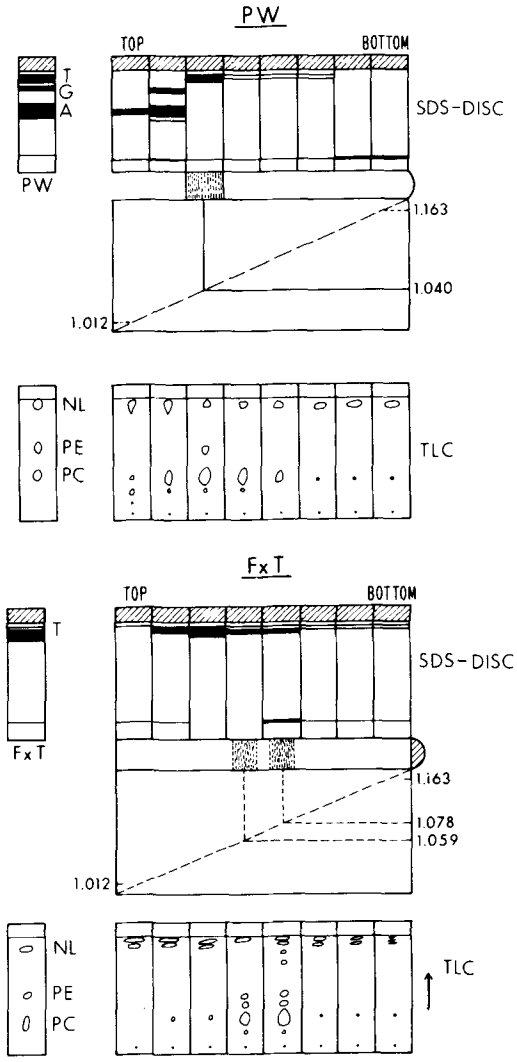

FIG. 2. Disc gel electrophoresis pattern of proteins and thin layer chromatography pattern of lipids from all the aliquots of the sucrose density gradient ultracentrifugation of rabbit pulmonary washing and Fraction T.

The phospholipid/protein ratios of the banded material varied between 1.0 and 3.0 (1.71 in Table I). But after 48 hr dialysis at 4 C,

TABLE I

Phospholipid (PL) and Protein Compositions of all the Aliquots from Sucrose Density Gradient Ultracentrifugations of (a) Rabbit Pulmonary Washing, (b) Fraction T and, (c) Fraction S

Density	Tube	Protein %	PL %	PL/Protein
(a) 1.012	1	21.0	9.3	0.57
1.025	2	26.2	14.1	0.70
1.040 (band)	3	24.6	32.5	1.71
1.057	4	7.0	27.0	5.16
1.074	5	5.5	13.0	3.00
1.091	6	3.0	2.0	0.89
1.117	7	4.4	0.8	0.25
1.162	8	8.7	1.7	0.25
(b) 1.020	1	3.4	0.0	0.00
1.028	2	27.9	3.8	0.14
1.043	3	35.4	5.7	0.17
1.059 (band)	4	17.0	43.9	2.77
1.078 (band)	5	8.8	42.5	5.17
1.097	6	1.7	1.1	0.70
1.123	7	1.7	1.4	0.86
1.169	8	4.1	1.4	0.35
(c) 1.016	1	3.7	3.1	5.00
1.028 (band I)	2	12.4	7.5	3.74
1.044 (band II)	3	17.2	21.1	7.65
1.059 (band III)	4	19.6	50.1	15.66
1.075	5	13.3	13.8	6.42
1.091	6	10.0	3.4	2.08
1.115	7	8.0	0.7	0.35
1.161	8	15.4	0.6	0.19

the ratio decreased considerably (e.g., from 1.71 to 1.07), because of loss of phospholipid during dialysis.

The other two major proteins, albumin and IgG, were found in the upper regions of the gradient with $\leqslant$ 23% phospholipid and appreciable quantities of neutral lipid (cholesterol > cholesteryl ester). Unlike the phospholipids, the neutral lipids were distributed nearly evenly throughout the gradient; all the free fatty acids were in the albumin fraction. Striking was the appearance of the small molecular weight protein in all the gradient fractions, particularly in the high density regions. The same pattern of lipid and protein distribution in the gradient was observed when 10x concentrated rabbit pulmonary washing was used.

(b) *Fraction T (Fig. 2, Table I):* Two wide bands were seen, a heavy one at d = 1.078 and a light band at d = 1.059. The combined bands contained 86% of the total phospholipid of Fraction T and only 26% of the protein. Most of the protein, sIgA (63%), was found in two lower density regions, 1.028 and 1.043, with little phospholipid and some neutral lipid (see TLC data). The small MW protein was also prominent in various regions of the gradient, and was concentrated in the higher density band.

(c) *Fraction S (Table I):* Three bands were observed, a major one (Band III) at d = 1.059 and two minor bands at densities 1.044 and 1.028. Most of the phospholipid (50%) was in the high density band together with 20% of the protein in a phospholipid/protein ratio of 16; the remaining protein was distributed more or less evenly throughout the gradient. Disc gel electrophoresis showed three proteins, albumin and γ-globulin in the upper regions of the gradient, and protein T mainly at densities > 1.044, in a pattern that was very similar to the one observed with pulmonary washing, in spite of the very large lipid/protein ratio in Fraction S.

Surface Activity

Because of known correlations between fatty acyl chain composition and surface tension lowering ability of lecithins (2,32-34), the total lipid was extracted from each of the ultracentrifugation bands of pulmonary washing, Fraction T (combined bands) and Fraction S, the fatty acyl composition was determined by gas liquid chromatography (Table II), and the dynamic surface tension was measured at 25 C with films that were spread from either the intact aqueous fraction or its total lipid extract. The surface tension vs. percent area data are

TABLE II

Fatty Acid Composition of Total Lipid Extracts of the Visible Bands from the Sucrose Density Gradient Ultracentrifugations of Pulmonary Washing, Fraction T and Fraction S (As Well as Lecithin Isolated from Rabbit Pulmonary Washing, in Extreme Left Column, PC).

				FxS Bands		
				I	II	III
Fatty acid	PC[a]	PW	FxT	d=1.028	d=1.044	d=1.059
	%	%	%	%	%	%
12:1(?)	---	2.2	---	---	---	3.3
14:0	2.4	2.6	2.8	9.7	2.2	1.8
14:1	0.3	0.9	0.4	1.4	0.4	1.1
16:0	68.3	67.4	69.7	69.6	58.7	67.4
16:1	7.3	4.6	4.0	3.2	3.7	5.1
18:0	1.6	2.9	2.5	---	2.1	3.4
18:1	12.5	14.2	14.1	1.8	23.5	14.6
18:x[b]	7.6	5.1	5.7	14.5	9.4	3.4
γmin	10.5	18.0	Zero	21.0	18.0	Zero
Factors %						
Positive						
($C_{16:0}+C_{18:0}$)	69.9	70.3	72.2	69.6	60.8	70.8
Negative						
(Total)	30.1	29.7	27.8	30.4	39.2	29.2

(?) Not identified.

[a]PC, phosphatidyl choline isolated from rabbit PW (Ref. 2).

[b]Probaly the *cis*-5-octadecenoic acid described by King et al. in dog lung (Ref. 43) and seen also by Colacicco and Scarpelli in rabbit pulmonary washing (Ref. 2).

presented in Figure 3.

The surface tension of saline, 72 dyne/cm, was not altered when the aqueous dispersion of the intact bands, containing 20 μg to 90 μg phospholipid was applied to the saline's surface; this meant that no lipid or protein spread as a film from the aqueous samples. However, when the same quantity of phospholipid was applied from the solution of the total lipid extract of the band in organic solvent, the surface tension was lowered to saturation values of 18 dyne/cm for pulmonary washing, zero for Fraction T and 21 dyne/cm, 18 dyne/cm and zero, respectively, for the low density (1.028), intermediate density (1.044), and high density (1.059) bands of Fraction S.

DISCUSSION

In the light of the foregoing data, we wish to focus our attention on five phenomena, which should be considered in the study of (a) the mechanisms of lipid and protein distribution in the preparation and ultracentrifugation of various surfactant fractions and (b) the physicochemical nature and behavior of pulmonary surfactant. The five phenomena are: The lipoprotein nature of pulmonary surfactant, the identity of the proteins of pulmonary washing, the isopicnic equilibrium of the major proteins of pulmonary washing, the small molecular weight protein, and the surface activity of the lipid extract of selected surfactant fractions.

Lipoprotein Nature of Pulmonary Surfactant

Under conditions in which lipoproteins are isolated from serum (2,26), virtually no protein was found in the lipid pellicle that floated on 1.090 g/ml KBr in differential ultracentrifugation of either pulmonary washing, Fraction T and Fraction S. Since less than 1% protein was found in the lipid pellicle and the protein consisted of traces of serum albumin and sIgA, we also conclude that no serum lipoprotein was present in rabbit pulmonary washing.

Our results are different from those of King and Clements (8,19), who, using a similar but not identical technique, found appreciable quantities (7 to 10%) of a small MW protein in the lipid pellicle. As it was pointed out by the other authors (Ref. 19, p. 724), such variations could be due to large as well as small differences in the experimental methods. Two speculations can be offered to explain the observed variations. First is the composition of the lavage

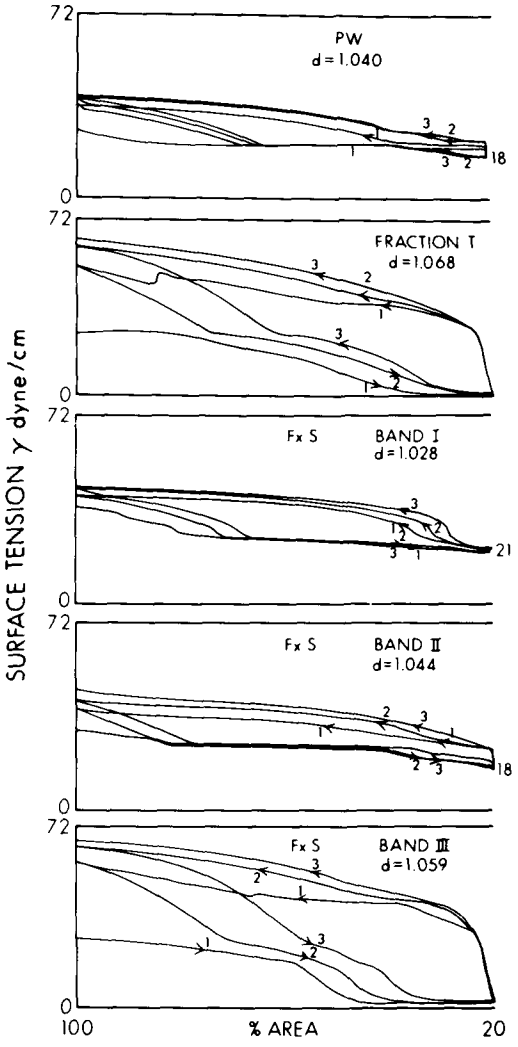

FIG. 3. Dynamic surface tension (γ)-area curves of spread films of total lipid extracts of the ultracentrifugation bands of pulmonary washing, Fraction T (combined bands; Fig. 2 and Table I), and Fraction S (Table I). Subphase: 0.15 M NaCl, 25 C.

medium; the presence of ions other than 0.15 M NaCl (8) could cause release of the specific pulmonary lipoprotein, which we left behind when the lavage medium was simply 0.15 M NaCl. Second, the various manipulations (repeated ultracentrifugations and dialyses; Ref. 8) could result either in the concentration of a given protein because of loss of lipid during dialysis or in the formation of an artifactual lipoprotein particle (31).

In the present study, the protein that could have been suspected to be associated with the phospholipid surfactant was protein T (5). However, several observations speak against the lipoprotein nature of such an association. First, it was recently found that protein T is an immunoglobulin A (6). Second, by immunofluorescence, the protein was not found in the alveolar lining layer (17). Third, a lipoprotein with a fixed stoichiometry would have banded in the gradient at a fixed density. Instead, with the same protein, densities and stoichiometries of the protein-lipid band were different, d = 1.040 and d = 1.070, depending on whether the starting material was pulmonary washing or Fraction T, respectively.

Our failure to obtain a specific lung lipoprotein in rabbit pulmonary washing does not negate the possible presence of lipoprotein in washings that were obtained and processed by different techniques. However, the identity of such lipoproteins (8,12,13,19) with pulmonary surfactant must still be demonstrated.

Identity of the Major Proteins of Pulmonary Washing and Fraction T and S

We have shown beyond doubt (above and Ref. 6) that the three major proteins of pulmonary washing (A, G, and T, in Fig. 2) are serum albumin, IgG, and IgA. We did not find the proteins of MW 36,000 or 62,000 which Bhattacharyya et al. described in rabbit pulmonary washing (13). It should be noted that the method used by the other authors (13) in preparing the lung lavage was quite different from ours. They discarded the washing that we collected in 0.15 M NaCl, and instead they studied a lavage that was obtained (after the 0.15 M NaCl washing) in hypotonic buffer; this medium conceivably dislodged (from the lung structures) constituents that we left behind.

For similar reasons, we may not be able to collect the fraction that was described by King et al. (11) and which contained a lung specific protein of MW 34,000. In the preparation of pulmonary washing, those authors used 5 mM tris and a few millimoles of $CaCl_2$ and $MgCl_2$ in their saline (0.15 M NaCl) buffered at pH 7.3 (8,9,11) as opposed to 0.15 M NaCl, pH ~5.6 in our experiments.

Isopicnic Equilibrium of the Major Proteins of Pulmonary Washing

Very striking was the position of serum albumin, IgG, and IgA at very low densities in the sucrose gradients of either pulmonary washing, Fraction T or Fraction S (Fig. 2 and Table I). Such a position cannot be accounted for simply by the average densities of the constituents. Consider, for example, two lipid-rich fractions, namely the band of pulmonary washing at d = 1.040 and the low density band

of Fraction T at d = 1.059. Using the data in Table I and Figure 2 for those bands and assigning a density of 1.35 g/ml to the hydrated protein (35) and 1.000 to 1.060 to the aqueous lipid (36-38), we obtain average densities (for the two bands) greater than 1.10 g/ml, not near the above values of 1.040 and 1.059. Furthermore, the tendency of proteins (albumin > IgG > IgA) to equilibrate in discrete regions of the sucrose gradient at densities smaller than 1.040 (Fig. 2 and Table I) must be accounted for by a new phenomenon. We propose a model in which the presence of small quantities of lipid causes the protein to attain conformations that make up an osmometer permeable to water.

The Small MW Protein

Negligible quantities of the protein of ca. 10,000 dalton were seen in the SDS disc gel electrophoresis of either pulmonary washing or Fraction T. Large amounts of this protein, however, appeared in the SDS gel of several aliquots from the sucrose density gradient ultracentrifugation (Fig. 2); since it was revealed abundantly also in the gradient aliquots of Fraction T, it seems as if originally this small protein was associated with sIgA. This suggestion must be substantiated by evidence.

It should be noted that this protein appears in markedly different regions of the gradient, depending on whether the starting material was pulmonary washing or Fraction T (Fig. 2). The data suggest that either the protein is not part of a native lipoprotein, or the latter's architecture and composition are altered during the various manipulations of the surfactant fractions (5,31,39).

The identity of the protein remains to be established. Its size suggests some similarity with the lung specific protein isolated by King et al. (9) from dog lung. However, another point should be made; if this protein is part of a lipoprotein in the band at density 1.040 in the sucrose gradient of pulmonary washing (Fig. 2, upper panel), it could not be the same lipoprotein described by King and Clements (8) at density 1.089, unless the two are altered differently in the two different procedures. The question will not be resolved until we verify the identity of the two proteins.

Surface Activity

The lecithin isolated from rabbit pulmonary washing was very active (γ_{min} = 0) when films were adsorbed from dispersions of 200 μg/ml in 0.15 M NaCl (2) and much less active (γ_{min} > 10 dyne/cm) when the film was spread from organic solvent (2,30). This difference could be caused by the physical state of the lipid (2,34,40), since it is conceivable that a large reservoir of lung lecithin in the subphase can provide a ready supply of molecular DPL to the film adsorbed at the interface, whereas in the spread film at 25 C most of the surface active DPL is eliminated after the first few compressions into surface structures that do not release molecular DPL (2,41).

Other factors, however, play a role; the molecular structure of lecithin is one. In the analysis of the activity of spread films of lecithin as compared to the other lipid extracts shown in Table II and Figure 3, we like to distinguish between positive and negative factors. Positive are the palmitoyl (16:0) and stearoyl (18:0) residues, since dipalmitoyl and distearoyl lecithins lower surface tension to zero at 25 C as well as at 37 C (33,34). Negative are cholesterol above 20 mole % (40) and all the unsaturated fatty acyl chains and the saturated ones with less than 16 C atoms.

Since the cholesterol content in all the examined fractions was very low (<5 mole % with respect to lecithin) and cholesteryl ester has no negative effect on the surface activity of DPL (40), the burden for the low surface activity seems to fall on the myristoyl chain (42) and all the unsaturated ones. At least two out of four such factors (14:0, 16:1, 18:1, 18:x) are high in those fractions whose spread films produced high γ_{min} values (>10 dyne/cm).

Except for band II of Fraction S, which had the lowest palmitoyl (58.7%) and the highest oleyl 23.5%) contents, all the other fractions contained about 70% positive factors, most of which was palmitoyl. Since the quantitative differences among the total positives or among the total negatives in the active (γ_{min} = zero) and inactive (γ_{min} > 10 dyne/cm) fractions are very small, a qualitative factor must be sought. This could be either the distribution of the acyl chains (dipalmitoyl vs. mixed palmitoyl-, oleyl, etc.), the specific effect of one unsaturated residue such as the *cis*-5-octadecenoyl (43) or some yet imponderable contaminant. Except for the surface activity effect of the mixed chain palmitoyl-myristoyl lecithin (42), little is known to answer the above questions. The negative effect of free fatty acids on the surface activity of DPL (44) must be excluded for two reasons; first, the ultracentrifugation bands did not contain free fatty acids, all of which were in the albumin fractions, second, the isolated lecithin (Table II and Ref. 2 and 30) which did not contain free fatty acids, was not active.

ACKNOWLEDGMENTS

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Effects of Ethionine and Phenobarbital on the Phosphatidylcholines of Rat Liver[1]

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ABSTRACT

The diglyceride species of phosphatidylcholines from livers of male rats after treatment of the animals with ethionine (1 mg/g divided among 4 doses 2 hr apart), phenobarbital (80 mg/kg each day for 3 days), or a combination of the two drugs were determined using gas chromatography. Ethionine treatment greatly elevated the diene species (significant at the 0.005 level for 34:2 and 0.001 level for 36:2). Phenobarbital treatment had no significant effect on the quantity of 34:2 but slightly increased that of 36:2 (significant at the 0.05 level). Both drugs caused relative decreases in the quantities of 38:4 (significant at the 0.001 level for ethionine and at the 0.01 level for phenobarbital). Ethionine decreased the content of 36:4 (significant at the 0.01 level) while phenobarbital treatment did not produce a significant effect on this fraction. Thus, while ethionine produced marked effects on the quantities of the various molecular species, the effects of phenobarbital were less dramatic. Combined treatment with both drugs generally produced levels of species similar to those produced by ethionine alone.

INTRODUCTION

Ethionine is known to inhibit S-adenosyl-L-methionine:phosphatidylethanolamine methyl transferase. This enzyme catalyzes the conversion of phosphatidylethanolamine to phosphatidylcholine by adding methyl groups donated by S-adenosyl methionine. This enzyme system has been shown by Arvidson (1) to be selective for phosphatidylethanolamines containing highly unsaturated fatty acids. The alternative route to phosphatidylcholine by way of the choline phosphotransferase pathway produces primarily phosphatidylcholines having one or two double bonds (2,3). Therefore, shutting down the methylation pathway should result in a population of phosphatidylcholines that contain less of the long-chain highly unsaturated acids (4).

The effects of phenobarbital on lipid metabolism are not so well understood. It is known that phenobarbital causes an increase in the liver endoplasmic reticulum, which requires an increase in phospholipid for proper structure of the newly formed endoplasmic reticulum. The increased hepatic phospholipid may result from either an increased rate of synthesis or a decreased catabolism (5) or both. Davison and Wills (6) demonstrated that 12 hr after rats were treated with phenobarbital the activity of the methyl transferase was increased, but that after 3 days, it was decreased. They also found that phenobarbital affected the fatty acid composition of the phosphatidylcholines irregularly: 18:2 increased about 20% while 20:4 and 22:6 were decreased.

We attempted to determine the effects of ethionine and phenobarbital on the molecular species of phosphatidylcholines in rat liver and to determine the molecular species by gas liquid chromatography.

MATERIALS AND METHODS

Male Charles River rats ca. 4-months-old obtained from our colony were sorted into matched groups of five animals each, according to weight, so that all groups contained animals of the same weight range.

Phenobarbital (Gotham Pharmaceutical Co., Inc., Brooklyn, NY) (162 mg/ml of propyleneglycol) was diluted with sterilized 0.9% saline to 40 mg/ml.

Ethionine (General Biochemicals, Chagrin Falls, OH) was dissolved in 0.9% saline to a concentration of 25 mg/ml.

Animals were fed Purina Rat Chow ad libitum until all food was removed.

One group of rats was injected intraperitoneally with phenobarbital at a dosage of 80 mg/kg each day for 3 days. A second group received, on the third day of the experiments, 1 mg DL-ethionine/g body weight divided among four doses 2 hr apart. The ethionine was administered by intraperitoneal injection. The third

[1] Structures of fatty acids and diglycerides are designated as follows: 18:2 or 36:4. The number before the colon is the number of carbons and the number after the colon is the number of double bonds. For the diglycerides, the number of carbons includes only the carbons of the constituent fatty acids and excludes those of glycerol and the trimethylsilyl ethers; the number of double bonds is the total in both fatty acids without regard for distribution.

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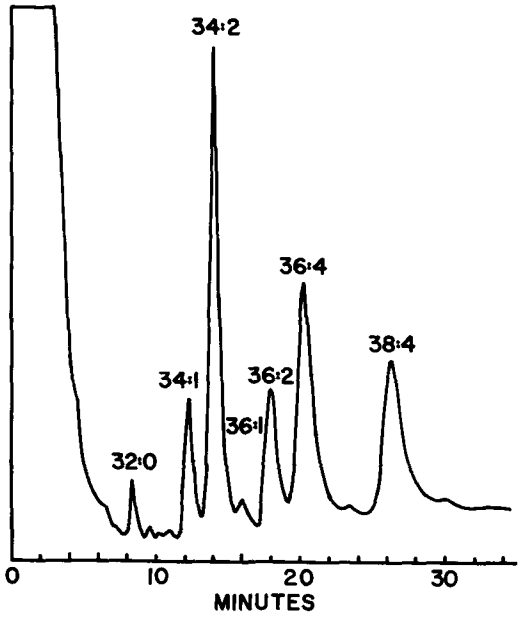

FIG. 1. Chromatogram of diglyceride trimethylsilyl ethers derived from the liver phosphatidylcholines from rats injected with saline or saline containing propyleneglycol. The phospholipids were extracted by the method of Folch et al. (7). Phosphatidylcholine was isolated using microcolumns of silica gel and preparative thin-layer chromatography. The trimethylsilyl ethers were prepared according to O'Brien and Klopfenstein (9) Gas liquid chromatography was on a Barber-Colman 5000 instrument using columns of 10% Apolar 10C on 100/120 mesh Gas Chrom Q. The columns were operated isothermally at 265 C.

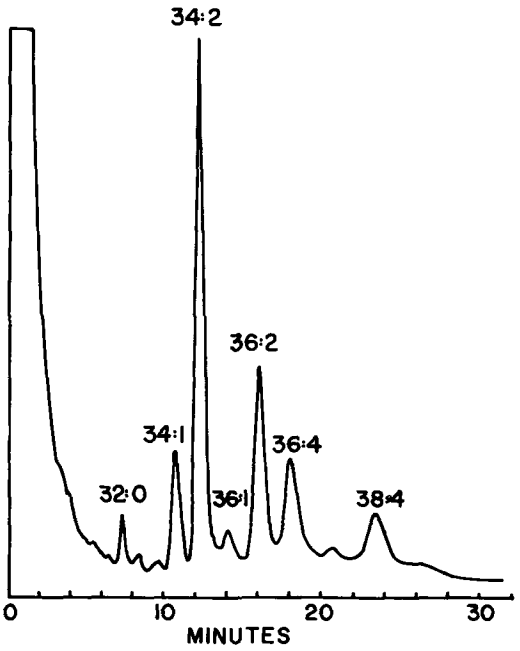

FIG. 2. Chromatogram of diglyceride trimethylsilyl ethers derived from the liver phosphatidylcholine of rats injected intraperitoneally four times at 2-hr intervals with a total of 1 mg DL-ethionine in saline/g body weight. (Samples were handled otherwise as in Fig. 1.).

group received both treatments. Of two groups of controls, one was injected with saline according to the ethionine schedule; the other received propyleneglycol in saline according to the phenobarbital schedule. Food was removed from all animals on the third day. The animals were decapitated on the fourth day and the livers removed, weighed, and frozen until the lipids were extracted by the method of Folch (7).

Lipids were fractionated on micro columns of silicic acid to remove neutral lipids followed by preparative thin layer chromatography (TLC) on basic silica gel using the solvent system of Skipski et al. (8). All TLC was carried out under a nitrogen atmosphere. The phosphatidylcholine bands were removed from the plates, and the phosphatidylcholine was eluted by repeated washes with 2:1 $CHCl_3$-CH_3OH.

The phosphatidylcholines were converted to diglycerides by hydrolysis by phospholipase C, and trimethylsilyl ethers were prepared from the diglycerides by treating them with N,O-bis-(trimethylsilyl) acetamide as described by O'Brien and Klopfenstein (9). The trimethylsilyl ethers were chromatographed on a Barber-Colman 5000 gas chromatograph using 8 ft x 3 mm columns packed with 10% Apolar 10C on 100/120 mesh Gas Chrom Q (Applied Science Laboratories, State College, PA). Columns were operated isothermally at 265 C. Trimethylsilyl ethers of commercial diglycerides (Nu-Chek Prep., Inc., Elysian, MN, and Serdary Research Laboratories, London, Ontario, Canada) were used as standards.

RESULTS AND DISCUSSION

A representative chromatogram of the diglyceride ethers derived from the phosphatidylcholines of one of the control rats (Fig. 1) indicated that the major components are well resolved by this system. The fatty acid components of the peaks were in order: 16:0,16:0; 16:0,18:1; 16:0,18:2; 18:0,18:1; 18:0,18:2; 16:0,20:4; and 18:0,20:4. No hexaene peak was apparent in the chromatogram, but on the basis of data from Land's laboratory (10), that fraction represented only 5% of the total phosphatidylcholines; furthermore, this 5% would be divided between 38:6 and 40:6 and thus would not be expected to produce major peaks.

Treatment with ethionine resulted in increases in the diene peaks and marked decreases in the tetraene peaks, as shown in Figure 2. That agrees with Arvidson's finding (1). Methyl transferase is selective for phosphatidylethanolamines containing highly unsaturated fatty acids. Because the enzyme is inhibited by ethionine, ethionine treatment would decrease the synthesis of the highly unsaturated phosphatidylcholines. The phosphotransferase pathway which produces phosphatidylcholines containing one or two double bonds (2,3) would continue to function unchecked. This would account for the observed increase of the less unsaturated species and for the decrease of the more highly unsaturated species in rats treated with ethionine.

Phenobarbital treatment resulted in increased 36:2 and decreased 36:4 and 38:4 relative to controls as is shown in Figure 3. That effect was in the direction of, but not so marked as, that produced by ethionine. A chromatogram of the diglycerides from rats treated with both drugs (Fig. 4) also shows increased levels of 34:2 and 36:2, characteristic of the ethionine treatment.

Quantitation of the chromatographic data (Fig. 5) shows 32:0 and 32:1 as minor species, representing less than 5% of the total diglycerides in all treatments. The diglyceride containing palmitic and oleic acids (34:1) is the first major component. That fraction of the phosphatidylcholines, though unchanged by the ethionine treatment, was depressed somewhat by phenobarbital, significant at the 0.05 level. The first diene fraction (34:2) was greatly elevated by the ethionine treatment, significant at the 0.005 level. Treatment with phenobarbital produced a quantity similar to that of the control. Treatment with both ethionine and phenobarbital produced a level elevated from the control, significant at the 0.01 level, but intermediate between that of either drug alone.

The monoene fraction of 36 carbons is also a relatively minor species which changes little with any of the drug treatments. On the other hand, 36:2 increased markedly on ethionine treatment, significant at the 0.001 level. Treatment with phenobarbital produced a level of 36:2 slightly above that of the control, significant at the 0.05 level. The two drugs combined produced a quantity of this fraction greater than that of the control, significant at the 0.005 level, and greater than that of the phenobarbital treated animals, significant at the 0.025 level, and not significantly different from that produced by the ethionine treatment alone.

The two tetraene fractions were affected similarly by ethionine treatment: decreases

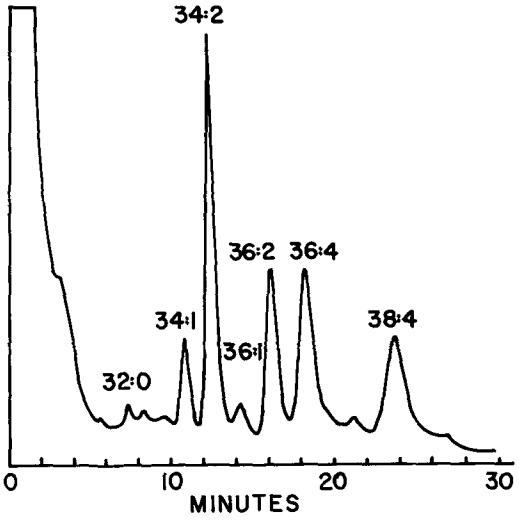

FIG. 3. Chromatogram of diglyceride trimethylsilyl ethers derived from the liver phosphatidylcholines of rats injected intraperitoneally with 80 mg phenobarbital in propyleneglycol-saline/kg body weight each day for 3 days. (Samples were handled otherwise as described in Fig. 1.)

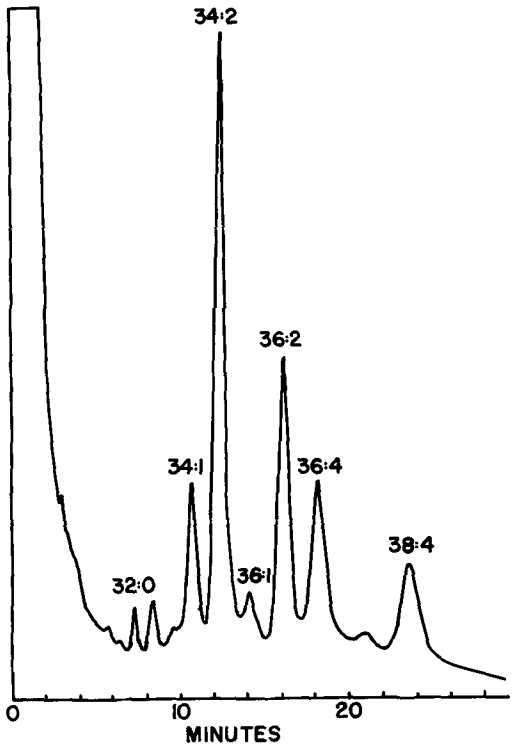

FIG. 4. Chromatogram of diglyceride trimethylsilyl ethers derived from the liver phosphatidylcholines of rats receiving both treatments (as described in Fig. 2 and 3).

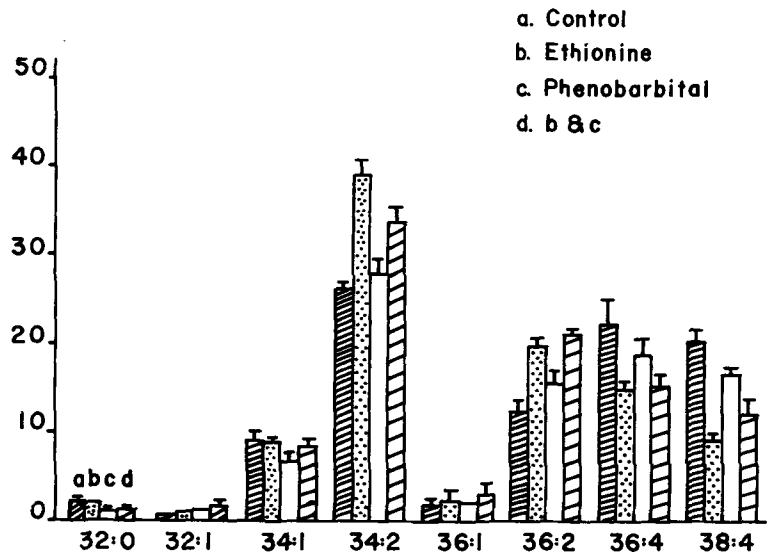

FIG. 5. Quantitation of diglyceride trimethylsilyl ethers obtained by the various treatments. The values are relative percentages, and lines above the bars represent the upper half of the standard deviations. Values are from four animals except for the double drug treatment for which the results are from three animals.

were marked in both, significant at the 0.01 level for 36:4 and at the 0.001 level for 38:4. Phenobarbital treatment also decreased the quantity of 38:4, significant at the 0.01 level. The apparent decrease in 36:4 on phenobarbital treatment is not significant. Treatment with both drugs produced levels of both tetraene species significantly decreased from control levels (0.01 level for both species) and not significantly different from the level produced by ethionine treatment alone.

The composition changes mentioned above are changes in relative composition. However, Björnstad and Bremer (11) stated that the total liver content of choline-containing phospholipids, of which phosphatidylcholine is the major species, was unchanged on induction of fatty liver by ethionine. Thus, the levels of phosphatidylcholine in livers of ethionine-treated rats should not be significantly different from those of control rats. Therefore, the changes in relative composition on ethionine treatment should represent actual changes in the contents in the liver of these species relative to control rats.

With phenobarbital treatment, the situation is more complex. In our experiments, we found that phenobarbital treatment caused an increase in the size of the liver of the treated rats to a ratio of liver wt/live rat wt of 0.038 compared to a ratio of 0.033 in the control animals. Thus, the phenobarbital caused a 15% average increase in liver mass. Acheampong-Mensah and Fener (12) recently reported data indicating that phenobarbital treatment for 5-7 days did not significantly change the quantity of total phospholipids per gram of liver tissue. They did, however, find a relative increase of 16% in the proportion of the total phospholipid which was phosphatidylcholine. Coupling this 16% increase in phosphatidylcholine per gram of liver with the 15% increase in the mass of the liver produces an increase of 33% in the phosphatidylcholine/liver on phenobarbital treatment. If a 33% increase in the total phosphatidylcholines is taken into account, the relative decreases in species 34:1, 36:4, and 38:4 on phenobarbital treatment disappear and the same total quantities of these species would be present in the phenobarbital-treated as there were in the control animals. Likewise, the increases in species 34:2 and 36:2 would become even more accentuated.

The rats receiving both drugs received phenobarbital for 2 days prior to ethionine treatment. Thus, the proliferation of smooth endoplasmic reticulum and concomitant increase in phosphatidylcholine characteristic of phenobarbital treatment is underway before ethionine treatment begins. Ethionine then causes its characteristic alteration of lipid metabolism and deposition of triglyceride. However, because of the phenobarbital-caused enlargment of the liver, more triglycerides can be deposited than with ethionine treatment alone. This scenario is supported by our observations of ratio of liver

wt/body wt. The observed ratio for the doubly-treated animals is 0.049 while that calculated by superimposing the ethionine-induced increase on that obtained for phenobarbital treatment is 0.048. Thus, it appears that the two drugs act independently on lipid metabolism. Therefore, the levels of total phospholipid and phosphatidylcholine in the doubly-treated animals should be the same as those in the phenobarbital-only-treated animals, since ethionine has been found not to alter these values (13).

The observed alterations of the molecular species of phosphatidylcholines with drug treatment are consistent with a decreased rate of methylation caused by ethionine administration resulting in a decrease in the content of the highly unsaturated species of phosphatidylcholine, which is synthesized primarily by that route. In addition to inhibiting methylation, ethionine reportedly stimulates phosphatidylcholine synthesis. Ulsamer and Glenn (14) found that, compared with untreated animals, ethionine treatment increased the incorporation of ^{32}P orthophosphate into phosphatidylcholine by way of the CDP-choline phosphotransferase pathway. Sato and Hasegawa (15) also found that ethionine increases the biosynthesis of phosphatidylcholine by that pathway. The increase in the Kennedy pathway by ethionine administration would explain the marked increase in the proportion of the less saturated phosphatidylcholines, species primarily synthesized by this route.

Because Young et al. (16) had reported that phenobarbital increased the specific activity of the S-adenosyl-methionine:phosphatidylethanolamine methyl transferase, we speculated that the diglycerides produced during phenobarbital treatment would contain a higher proportion of the more highly unsaturated species. That did not occur, as is evident in Figure 3. We had administered phenobarbital for 3 days. Davison and Wills (17) have reported that, at 3 days, phenobarbital produces a decreased level of methyltransferase activity. This would cause the quantity of the polyene species to decrease, which was observed by us in our experiments.

Additionally, many investigators have established that phenobarbital decreases the catabolism of phosphatidylcholine. Arvidson (1) observed that, under normal conditions, the hexaene phosphatidylcholines were degraded most rapidly. Decreased catabolism would result in a stabilization of the highly unsaturated species. Thus, phenobarbital could exert opposite effects of decreasing the formation of the highly unsaturated species and decreasing catabolism, thus producing levels of those species little changed from control levels. This is as we have observed. Although the relative concentrations of the polyene species decreased with phenobarbital treatment, the total quantity of phosphatidylcholine in a liver could have increased ca. 33%, sufficient to convert the relative decreases into no change in the total quantities of those species present.

The intermediate values of the combined phenobarbital plus ethionine treatment can also be explained by the same hypothesis. Even though both drugs decrease the methylation of the polyene phosphatidylethanolamines, the added effect of the phenobarbital on the catabolic enzymes would prevent the levels of those species from falling to the levels observed with ethionine treatment alone.

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Hydrocarbon Gases Produced During In Vitro Peroxidation of Polyunsaturated Fatty Acids and Decomposition of Preformed Hydroperoxides

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ABSTRACT

Hydrocarbon gases have been used previously as an index of lipid peroxidation in vivo and in vitro. In vitro experiments are reported on the formation of hydrocarbon gases from peroxidizing ω-3 and ω-6 fatty acids. Hydrocarbon gases were not released during a 20-hr peroxidation phase but were released following the decomposition of hydroperoxides by addition of excess ascorbic acid. The major hydrocarbon gas products in iron, copper, or hematin catalyzed peroxidation systems were ethane or ethylene from linolenic acid, and pentane from linoleic acid and arachidonic acid. Calculations of the ratios of hydrocarbon gases formed were based on fatty acid decrease and/or change in diene conjugation and peroxide values. Depending on the fatty acid, catalyst, and calculation basis used, pentane formation was as high as 1.3 mol %, ethane 4.3 mol %, and ethylene 10.6 mol %.

INTRODUCTION

There are a number of methods used for the measurement of lipid peroxidation in vivo and in vitro; e.g., determination of peroxide value, diene conjugation, malonaldehyde and fluorescent products. Since the appearance of hydrocarbon gases during the autoxidation of fats was first noted (1), several papers have described the measurement of these products to follow lipid peroxidation. Saturated hydrocarbon gases were shown to arise early during autoxidation of soybean oil and methyl linoleate (2,3). Pentane was the predominant short chain hydrocarbon gas product noted to arise through thermal decomposition of lipoxidase derived products of linoleic acid (4), and several low molecular weight hydrocarbon gases were found in oxidized butterfat (5). Correlations of flavor scores and pentane formation were used to determine rancidity of oils (6-8). In dehydrated food systems, oxidation processes were measured by thermal release of hydrocarbons, and several predicted hydrocarbon gases were found (9).

Plaa and Witschi (10) concluded that the concept of lipid peroxidation in vivo is one of the important concepts of current experimental pathology and toxicology, in spite of the fact that some of the most convincing evidence for the role of lipid peroxidation is indirect and that many conclusions have been drawn by inference. Most conclusions have been based on the finding of increased malonaldehyde levels in tissues (11) and on the presence of fluorescent products that arise in part by malonaldehyde reaction with other biological compounds (12). Ethylene formation was shown to be coupled with the peroxidation of lipids in rat liver microsomes in a cuprous-generating system (13). It was suggested (14) that "activated" linolenic acid was the possible source of ethylene. Ethane production was shown to be characteristic of lipid peroxidation in vivo (15-17). Recently, pentane was shown to be an even more sensitive index of lipid peroxidation in rats fed a vitamin E-deficient diet (18) and additionally stressed by exposure to ozone (19).

Earlier studies (18,19) showed that the measurement of hydrocarbon gases is advantageous in that it is a nondestructive method that is applicable to experiments in vivo and that it is an easy and direct measurement of lipid peroxidation products. It was the purpose of this study to quantitate the hydrocarbon gases produced from iron and copper catalyzed decomposition of hydroperoxides.

EXPERIMENTAL PROCEDURES

Materials

Methyl linoleate and methyl arachidonate were obtained from The Hormel Institute, Austin, MN, and methyl linolenate from Analabs, North Haven, CT; all were >99% pure and were used without further purification. Hydroperoxides of the three fatty acid methyl esters were prepared by the procedure of O'Brien (20). The peroxidation time was 4-7 days. The last purification step by preparative thin layer chromatography was omitted.

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Peroxidation of Fatty Acid Methyl Esters and Decomposition of Hydroperoxides

Erlenmeyer flasks (25 ml) with rubber stoppers were used as reaction vessels. The 3-ml reaction mixture contained: 0.2 ml of 170-180 mM methyl linoleate, methyl linolenate, or methyl arachidonate in methanol that contained methyl pentadecanoate as an internal standard; 0.2 ml of 10 mM $FeCl_3$, $CuSO_4$ or hematin; 0.2 ml of 20 mM ascorbic acid; and 2.4 ml of 0.1 M potassium phosphate buffer, pH 7.0, in 1% Brij 35. After incubation with shaking at 37 C for 20 hr, an additional 0.2 ml of 360 mM ascorbic acid was added to reduce the cations that mediate decomposition of hydroperoxides. The blanks contained methanol without fatty acids. Samples of 0.25 ml were removed prior to incubation, after 20 hr and 20 min following the addition of the second aliquot of ascorbic acid. The samples were immediately added to 1.25 ml of butanol and stored at -20 C for subsequent analysis of fatty acids, diene conjugation, and peroxide value. Parallel headspace gas samples taken with a gastight syringe were analyzed immediately via gas chromatography.

Decomposition of Preformed Hydroperoxides

A 0.2-ml sample that contained a known concentration (60-100 mM) of the different fatty acid methyl ester hydroperoxides in methanol was mixed with 2.4 ml 0.1 M potassium phosphate buffer, pH 7.0, 0.2 ml of 10 mM $FeCl_3$, $CuSO_4$, or hematin, and 0.2 ml of 360 mM ascorbic acid added via a syringe through the rubber stopper. Headspace gases were analyzed before and after addition of catalyst and ascorbic acid.

Analytical Methods

Fatty acid methyl esters were measured in a Packard model 427 gas chromatograph equipped with a flame ionization detector and a glass column (6 ft x 2 mm) packed with 3% OV-1 on 80/100 Supelcoport. A nitrogen carrier gas flow rate of 20 ml/min was used. The injector and detector temperatures were 260 C and 300 C, respectively. The column temperature was programmed as follows: 190 C for 1 min, followed by a 39 C rise/min to a temperature of 240 C, which was held for 1 min. The fatty acid methyl esters were quantitated on a basis of the methyl pentadecanoate internal standard.

Conjugated diene measurements of peroxidized fatty acids were made after dilution of the samples in hexane. Further dilution of the samples was required to avoid interference by ascorbic acid. The conjugated dienes of preformed hydroperoxides were determined on a methanolic solution. Calculations were based on an extinction coefficient of $\epsilon_{232} = 25000$ $M^{-1}cm^{-1}$ (21).

Fatty acid hydroperoxides were determined by a photometric method (22). Sample aliquots of 0.5 ml were added to 6 ml chloroform-acetic acid (1:2) at 37 C and were constantly deaerated by bubbling a stream of nitrogen through the solution. A 0.1-ml aliquot of half-saturated KI solution was added. After 10 min, the volume was adjusted to 10 ml with methanol and mixed well. Following dilution of the sample 1:10 with methanol, the absorption was measured immediately at 370 nm. The conversion factor used for calculation of the peroxide content was verified by comparison with the results of an enzymatic measurement of linoleic acid hydroperoxide (23). One microequivalent of iodine, equal to 1 microequivalent of peroxide, gave an absorption of 0.385.

For hydrocarbon gas analysis, a Varian-Aerograph model 1520 gas chromatograph with a flame ionization detector was used. A stainless steel column (1/8 in. x 5 ft) filled with activated alumina (80-100 mesh) was used with a nitrogen carrier gas flow rate of 25 ml/min. The injector temperature was 165 C, and the detector temperautre was 265 C. The column temperature was programmed at 70 C for 1 min followed by a 30 C rise/min to 250 C. The total program was for 8 min. Headspace gas samples of 0.25-0.5 ml were injected, and the relative peak areas of the hydrocarbon gases were calculated from pentane and ethane standards (Matheson Gas Products, Newark, CA). A mixed gas standard (C1 to C6, 15-20 ppm, Scott Environmental Technology, Inc., Plumsteadville, PA) was used to quantitate the other hydrocarbon gases.

RESULTS

Hydrocarbon Gas Formation after Peroxidation of Fatty Acid Methyl Esters and after Decomposition of Hydroperoxides

Peroxidation of methyl linoleate, methyl linolenate, and methyl arachidonate was catalyzed with iron, copper, or hematin. Using the decrease in fatty acid as a measure of peroxidation, the data presented in Table I show that hematin was the most effective catalyst for methyl linoleate and that copper was most effective as a catalyst for peroxidation of methyl linolenate and methyl arachidonate. The overall decrease of fatty acids varied from 53 to 98% in 20 hr.

Diene conjugation and peroxide values in the

TABLE I

Fatty Acid Decrease and Products of Iron, Copper, and Hematin Catalyzed Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Fatty acid decrease (μmoles)	Diene conjugation (μmoles)	Peroxide value (μmoles)	Major hydrocarbon gas	
					Ethane (nmoles)	Pentane (nmoles)
Linoleate	Fe	23.3	12.6	16.5		121.7
	Cu	17.6	11.4	11.7		105.2
	Hematin	25.4	6.4	4.2		5.0
Linolenate	Fe	19.2	7.2	10.4	210.9	
	Cu	27.5	7.0	9.1	301.6	
	Hematin	20.9	2.2	6.6	3.2	
Arachidonate	Fe	22.9	8.0	11.0		91.2
	Cu	35.7	5.7	5.8		19.2
	Hematin	23.6	4.8	2.0		1.9
	Hematin[b]	27.4	4.1	3.0		9.3

[a]The reaction mixtures, containing 35-36 μmoles of fatty acid, and conditions are described in Experimental Procedures. Fatty acids, diene conjugation, and peroxide values were measured prior to decomposition and hydrocarbon gases following decomposition of hydroperoxides initiated by excess ascorbic acid. Each value is the average of data obtained from five different reaction mixtures.

[b]Excess ascorbic acid was added at the beginning of the incubation period.

TABLE II

Ethane and Pentane Evolved Following Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Major hydrocarbon gas	Major hydrocarbon gas relative to:		
			Fatty acid decrease (mol %)	Diene conjugation (mol %)	Peroxide value (mol %)
Linoleate	Fe	Pentane	0.5	1.0	0.7
	Cu	Pentane	0.6	0.9	0.9
	Hematin	Pentane	<0.1	0.1	0.1
Linolenate	Fe	Ethane	1.1	2.9	2.0
	Cu	Ethane	1.1	4.3	3.3
	Hematin	Ethane	<0.1	0.1	<0.1
Arachidonate	Fe	Pentane	0.4	1.1	0.8
	Cu	Pentane	0.1	0.3	0.3
	Hematin	Pentane	<0.1	<0.1	0.1
	Hematin[b]	Pentane	<0.1	0.2	0.3

[a]The reaction mixtures are described in Experimental Procedures. Except where indicated, hydrocarbon gases were measured in headspace gases following 20 hr of incubation and addition of excess ascorbic acid.

iron and copper catalyzed systems show that hydroperoxides are the major products after 20 hr of peroxidation. The larger decreases in fatty acid compared with increases in diene conjugation and peroxides indicate that some decomposition of the hydroperoxides occurred during the 20 hr incubation. The extent of decomposition was greater in the fatty acids with the greatest degree of unsaturation, indicating that the hydroperoxides with less unsaturation were more stable. In the hematin catalyzed systems, diene conjugation and peroxide values were much lower than in the iron and copper catalyzed systems even though the decreases in fatty acids were in a similar range.

During the 20 hr of incubation to peroxidize the fatty acids, the production of hydrocarbon gases was negligible. The hydrocarbon gases analyzed were methane, ethane, ethylene, propane, butane, pentane, and hexane. However, the methane peak could not be quantitated because it was too close to the air pressure peak following sample injection. By estimation, methane was noted to be in the range of other minor hydrocarbon gases. Hexane was not detected in the samples because it was either not released or not volatile enough at 37 C. The amounts of hydrocarbon gases shown in Table I are those measured after the addition of excess ascorbic acid which resulted in rapid evolution of the gases. Since it is known that hydroperoxide decomposition by Fe^{3+} or Cu^{2+} is

TABLE III

Minor Hydrocarbon Gases Evolved Following Peroxidation of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester	Catalyst	Major hydrocarbon gas	Butane (mol %)	Propane (mol %)	Ethylene (mol %)	Ethane (mol %)
Linoleate	Fe	Pentane	16.3	6.4	<1.0	5.0
	Cu	Pentane	14.5	2.4	<1.0	2.1
Linolenate	Fe	Ethane	0	0	<1.0	
	Cu	Ethane	0	0	46.3	
Arachidonate	Fe	Pentane	13.5	1.7	<1.0	5.2
	Cu	Pentane	27.6	8.4	7.2	15.9

[a]The reaction mixtures are described in Experimental Procedures. Hydrocarbon gases were measured following addition of excess ascorbic acid after 20 hr of incubation. Calculations of mol % are expressed relative to the major hydrocarbon gas evolved.

TABLE IV

Hydrocarbon Gases Evolved During Iron, Copper, and Hematin Catalyzed Decomposition of Preformed Hydroperoxides of Methyl Linoleate, Linolenate, and Arachidonate[a]

Fatty acid methyl ester hydroperoxide	Catalyst	Major hydrocarbon gas	Major hydrocarbon gas relative to: Diene conjugation (mol %)	Peroxide value (mol %)
Linoleate	Fe	Pentane	1.3	1.3
	Cu	Pentane	0.7	0.6
	Hematin	Pentane	0.3	0.3
Linolenate	Fe	Ethane	3.0	2.3
	Cu	Ethylene	10.6	8.3
	Hematin	Ethane	0.4	0.3
Arachidonate	Fe	Pentane	0.9	0.7
	Cu	Pentane	0.5	0.4
	Hematin	Pentane	0.3	0.2

[a]The reaction system for decomposition of preformed hydroperoxides is described in Experimental Procedures. Data shown represent the average of values obtained for analysis of three separate reaction systems.

markedly stimulated by a hydrogen donor like ascorbic acid (20), it is assumed that the formation of hydrocarbon gases occurs during the decomposition of hydroperoxides only. The amount of hydrocarbon gases formed in the hematin catalyzed reactions was much smaller than that formed in the iron and copper catalyzed systems. As shown in Table I, the amount of pentane formed from methyl arachidonate in the hematin catalyzed system was greater when higher amounts of ascorbic acid were added at the beginning of the peroxidation. In this system, pentane was measured at intervals during the 20-hr incubation and was found to be evolved continuously. The increased formation of pentane was probably dependent upon maintenance of the reduced state of the iron in the hematin.

The major hydrocarbon gases formed were ethane from methyl linolenate and pentane from methyl linoleate and methyl arachidonate. The ratios of these gases formed following decomposition of the hydroperoxides are shown in Table II. Depending on the fatty acid, catalyst, and calculation basis, pentane formation was as high as 1.1 mol % and ethane, 4.3 mol %. Since hydroperoxides are not the only peroxidation products, as shown in Table I, the ratios of hydrocarbon gases produced based on the decrease of fatty acid were always smaller than those based on diene conjugation or peroxide value.

The amounts of other low molecular weight hydrocarbon gases that were detected, relative to the major hydrocarbon gas products, are shown in Table III. In the copper catalyzed methyl linolenate systems, about 46% as much ethylene was detected as ethane, and in the methyl linoleate and methyl arachidonate systems, between 13.5 and 27.6% as much butane as pentane was produced. Other hydrocarbon gases were only of minor importance. Measurement of the peroxidation reaction by following the decrease in fatty acid showed that continuation of peroxidation during the decomposition of hydroperoxides by excess ascorbic acid for

20 min was negligible. Small amounts of ethane and ethylene formed by decomposition of Brij 35, a polyethylene ether of lauryl alcohol, were corrected for by the use of appropriate blanks.

Hydrocarbon Gas Formation Following Decomposition of Preformed Hydroperoxides of Fatty Acid Methyl Esters

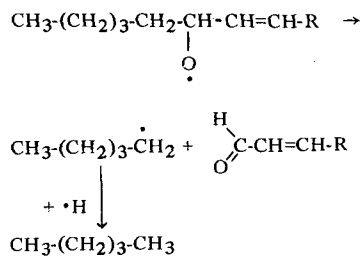
After it was found that hydrocarbon gases were formed only during the decomposition of the fatty acid hydroperoxides, similar assays were done with known amounts of preformed hydroperoxides. Table IV shows that with one exception the ratios of hydrocarbon gases in the total system are similar to those shown in Table II. The ratio of ethane:ethylene formed in the copper catalyzed methyl linolenate hydroperoxide decomposition systems was 2:1 in the total peroxidizing system and 1:12 in the preformed hydroperoxide system.

DISCUSSION

This investigation of the use of the low molecular weight hydrocarbon gases as indices of lipid peroxidation was initiated following the reports that ethane production in vivo was related to treatment of mice with carbon tetrachloride (15), and that measurement of ethane and pentane was used to show protection by vitamin E against lipid peroxidation in rats (18). Further literature references show that many volatile compounds have been measured from peroxidizing food systems. Pentane evolved from peroxides during their decomposition has been correlated with sensory tests for rancidity (6-8), and it also has been measured as a decomposition product of 13-hydroperoxy-octadeca-9,11-dienoic acid, a lipoxidase oxidized product from linoleic acid (4,24). In dehydrated food systems where oxidation was measured by thermal release of hydrocarbons, the following gas products were found: ethane from linolenic acid; octane from oleic acid; pentane from linoleic acid; pentane and butane from myristoleic acid; and heptene and hexane from vaccenic acid (9).

This report shows that the hydrocarbon gases are evolved only during the decomposition of fatty acid hydroperoxides and not during the peroxidation phase. The major hydrocarbon gas products are pentane, which arises from ω-6 fatty acids, and ethane or ethylene, which arise from ω-3 fatty acids.

The general route for formation of ethane and pentane from free radical products of hydroperoxide decomposition has been visualized by Evans et al. (4). The main reaction of β-scission is well-known for oxy radicals from decomposing peroxides (25-27). β-Scission is the production of a stable molecule by unpairing of the electrons in the bond which is β to the free radical. The most likely precursor for pentane formation is an alkoxy radical that undergoes β-scission very readily. Cleavage of an ω-6 fatty acid chain on the side of the peroxy group closest to the methyl end results in the formation of a pentane free radical as shown in reaction (I). Hydrogen abstraction by the resulting free radical forms pentane.



(I)

This study demonstrates that pathway (I) is even more likely once the route of transition metal-catalyzed decomposition of hydroperoxides is considered:

$$ROOH + M^{+n} \rightarrow RO\cdot + OH^- + M^{+n+1} \quad \text{(II)}$$

$$ROOH + M^{+n+1} \rightarrow ROO\cdot + H^+ + M^{+n} \quad \text{(III)}$$

In their lower but not in their higher oxidation states, the iron and copper catalysts used in these experiments are effective reducers of ROOH (28). Iron, hematin, and copper are the metals that decompose hydroperoxides and are present in higher concentrations in vivo when compared to other heavy metals. Therefore, it can be assumed that alkoxy radicals are major decomposition intermediates that eventually lead to release of pentane or ethane by β-scission.

The amount of hydrocarbon gases formed in the hematin catalyzed reactions was much smaller than that formed in the iron and copper catalyzed systems. This was probably dependent upon the fact that the reduced state of iron in hematin is harder to achieve than it is in free iron ions. Hematin is known to be a very effective inducer of lipid peroxidation, and contrary to free ions, it is a potent decomposer of hydroperoxides (20,29). For these reasons, it is assumed that the decomposition reaction mainly follows route (III).

It would be interesting to account for the origin of the minor hydrocarbons, butane, propane, ethane, and ethylene, that derive from

the ω-6 fatty acid hydroperoxides. Considering that β-scission is a predominant route of scission reactions (25-27), the origin of these minor hydrocarbons might be visualized as follows. β-Scission of the 13-hydroperoxyl radical (III) would yield the 13-alkyl radical, and β-scission of this radical would yield butane radical. A β-scission of butane radical gives ethylene and ethane radical. In a similar manner, β-scission of pentane radical of (I) would give ethylene and propane radical. Hydrogen abstraction by these alkyl radicals gives the alkanes, butane, ethane, and propane.

It is assumed that the general decomposition mechanism in the ω-3 fatty acid systems also follows reaction (I), but gives rise to ethane. Copper is known to react with alkyl radicals to give the corresponding a alkene (25). This reaction (IV) can account for the formation of ethylene.

$$C_2H_5^{\circ} + Cu^{++} \rightarrow CH_2{=}CH_2 + H^+ + Cu^+ \qquad (IV)$$

Ethylene was produced in larger amounts from preformed hydroperoxides, but the conditions favoring results of this are not apparent.

Only a qualitative statement about the relationships among conjugated dienes, peroxide values, and hydrocarbon gases can be given. Hydrocarbon gases can be produced upon the addition of ascorbic acid only after the appearance of conjugated dienes and peroxides, and they are not detectable when conjugated dienes and peroxides are negligible. Earlier studies by Jarvi et al. (7) showed a close correlation between peroxide values and pentane released after thermal decomposition of soybean oil hydroperoxides.

The calculated molar ratios show that hydrocarbon gases are only minor lipid peroxidation products compared to those obtained in studies by Chan et al. (30), where on a molar basis a 67-80% yield of volatile cleavage products of thermally decomposed methyl linoleate hydroperoxide was found in the form of hexanal, methyl octanoate, 2,4-decadienals, and methyl 9-oxononanoate. A major usefulness of the molar ratios determined is that they can be applied to measurements of pentane and ethane produced during lipid peroxidation in vivo. These ratios allow a calculation of the approximate amounts of fatty acid hydroperoxides formed and decomposed in vivo. The advantages of the measurement of hydrocarbon gases as products of lipid peroxidation are that they are volatile at ambient temperature, that they are stable and chemically inert, and that they are easily detectable in minimal amounts both in vivo and in vitro.

ACKNOWLEDGMENTS

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: I. Methyl Oleate[1]

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ABSTRACT

A structural investigation of autoxidation products of methyl oleate was carried out by gas chromatography-mass spectrometry (GC-MS) of trimethylsilyl (TMS) ether derivatives. GC-MS using computer plots of selected masses afforded structural assignments of GC peaks due to incompletely resolved mixtures. This method provided evidence of epoxy and keto esters which are not completely separated from the main components consisting of the TMS derivatives of the allylic hydroxy esters. Use of an MS-computer system also showed that the hydroxyoctadecanoate TMS ethers were partially separated by GC. The use of synthetic hydroxyoctadecanoates for the first time enabled us to demonstrate the quantitative reliability of a GC-MS computer summation approach to analyze the isomeric composition of oleate hydroperoxides (as the saturated TMS ether derivatives). Consistently higher concentrations were found of the 8- and 11-hydroperoxides than of the 9- and 10-hydroperoxides. Minor products of autoxidation identified by GC-MS include allylic enones, isomeric epoxyoctadecanoates, dihydroxyoctadecenoates, and dihydroxyoctadecanoates.

INTRODUCTION

Autoxidation of unsaturated fatty acids decreases the biological availability of these essential dietary components of vegetable oils and produces not only various flavor deteriorations in foods but also potentially toxic and unsafe materials during processing and cooking. Peroxidation of unsaturated fats in vivo causes damage to membranes, enzymes, proteins, and other lipids and vitamins. For these reasons, there has been renewed interest in our laboratory and elsewhere in the problems of fat autoxidation (1). Partial hydrogenation has improved oil stability considerably but has not solved the problem completely. Better ways than hydrogenation are needed to stabilize vegetable oils, and soybean oil in particular, against oxidation. Basic research in this field will increase our knowledge of the factors involved in preventing flavor deterioration due to autoxidation of unsaturated fatty acids.

Although much work has been carried out on the autoxidation of oleic acid and related compounds, there is confusion in the literature on the relative distribution of isomeric hydroperoxides, the effect of different reaction conditions, and the nature of secondary products. The present work was aimed at throwing new light on these questions by using gas chromatography-mass spectrometry (GC-MS) to study the isomeric distribution of allylic hydroperoxides and some of the secondary products which may serve as nonvolatile precursors of off-flavors.

GC-MS of trimethylsilyl (TMS) ether derivatives has been used in a number of structural investigations of oxygenated fatty acid derivatives (2), autoxidized fatty esters (3,4), and acetoxylated methyl oleate (5). Although this method has proved to be a powerful tool in qualitative studies, much care is needed when it is applied for quantitative work. Our studies now carried out for the first time with authentic samples of isomeric hydroxyoctadecanoate indicate that misleading results can be easily obtained in this way. Careful summation of mass spectra by computer is essential. The quantitative reliability of the GC-MS computer-summation approach was demonstrated with known mixtures of synthetic 8-, 9-, 10-, and 11-hydroxyoctadecanoates for autoxidized oleate. Since the oxidation products of natural fats include hydroperoxides of oleate, linoleate, and linolenate, methods to determine their origin would be of considerable importance. In subsequent papers of this series we use GC-MS to analyze autoxidized linoleate, linolenate, and their mixtures with oleate, and to determine how their hydroperoxides interact during autoxidation. In other papers we shall describe the application of high pressure liquid chromatography and the stereochemistry of the various autoxidation products.

EXPERIMENTAL PROCEDURES

Methyl oleate was prepared from commer-

[1]Presented at the AOCS Meeting, Chicago, September 1976.

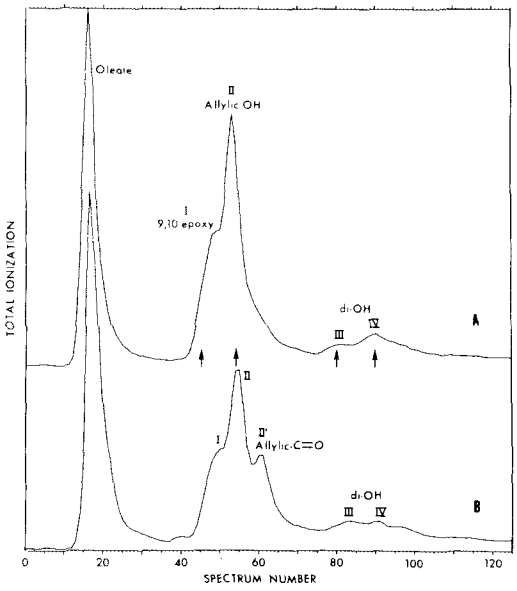

FIG. 1. GC-MS of silyl ethers of reduced-autoxidized methyl oleate (PV 1370). A. $NaBH_4$-reduced; B. KI-reduced.

cial oleic acid (Pamolyn-100, Hercules, Inc, Wilmington, DE) and purified by counter double current distribution (6), silicic acid chromatography, and vacuum distillation. It analyzed 98.0% by GLC (impurity: 2% stearate).

Autoxidations were done with 2 to 5 g oleate stirred under O_2 in a 50-ml Erlenmeyer flask attached to a manometric system and immersed in a temperature-controlled bath. Peroxide values were determined idometrically (7). The KI-reduced lipid products were isolated from the separated $CHCl_3$ layer and combined with the diethyl ether extracts from the aqueous layer. $NaBH_4$ reduction was done in aqueous MeOH (8). Catalytic hydrogenations were carried out at atmospheric pressure on 0.5-g samples in 95% EtOH with PtO_2. Hydrogen uptake was followed manometrically and continued 10 to 15 min after absorption ceased.

Hydroxy ester derivatives were silylated with bis(trimethylsilyl) trifluoroacetamide (Regis Chemical Co., Morton Grove, IL). GC-MS analyses were carried out by the same procedure described previously (5). The GC was per-

TABLE I

Mass Spectral Data

Peak	Spectrum no.	Characteristic fragments m/e (relative abundance)	Identification[a] (methyl ester)
I		1. Reduced-oxidized oleate (Fig. 1A)	
I	45	155(90.5), 171(17.5), 199(12.7)	9,10-Epoxyoctadecanoate
II	54	227(100)	9-OH,10-Octadecenoate (OTMS)
		241(88.7)	8-OH,9-Octadecenoate (OTMS)
		271(80.9)	10-OH,8-Octadecenoate (OTMS)
		285(76.4)	11-OH,9-Octadecenoate (OTMS)
III	80	243(15.4), 245(31.1)	8,9-diOH-Octadecenoate (OTMS)
		257(20.2), 259(36.9)	9,10-diOH-Octadecenoate (OTMS)
		271(10.5), 273(7.4)	10,11-diOH-Octadecenoate (OTMS)
IV	90	245(4.7), 229(2.9)	8,9-diOH-Octadecanoate (OTMS)
		259(35.9), 215(35.9)	9,10-diOH-Octadecanoate (OTMS)
		273(2.1), 201(10.7)	10,11-diOH-Octadecanoate (OTMS)
		2. Hydrogenated-oxidized (Fig. 3)	
I	15	155(84.6), 171(17.7), 199(15.1)	9,10-Epoxyoctadecanoate
	19	169(15.6), 185(15.2), 157(19.4)	8,9-Epoxyoctadecanoate
		155(40.6), 171(32.4), 199(15.3)	9,10-Epoxyoctadecanoate
		141(21.4), 213(8.9), 185(15.2)	10,11-Epoxyoctadecanoate
II	31	243(30.0), 245(42.9)	8-OH-Octadecanoate (OTMS)
		229(34.3), 259(38.7)	9-OH-Octadecanoate (OTMS)
		215(41.1), 273(37.3)	10-OH-Octadecanoate (OTMS)
		201(56.2), 287(44.5)	11-OH-Octadecanoate (OTMS)
III	70	245(51.9), 215(80.5)	8,10-diOH-Octadecanoate (OTMS)
		245(51.9), 201(65.2)	8,11-diOH-Octadecanoate (OTMS)
		259(79.9), 215(80.5)	9,10-diOH-Octadecanoate (OTMS)
		259(79.9), 201(65.2)	9,11-diOH-Octadecanoate (OTMS)

[a]Based on comparisons with reference compounds: methyl 9,10-epoxyoctadecanoate, 8-, 9-, 10-, and 11-hydroxyoctadecanoate, 9,10-dihydroxyoctadecanoate and reported fragmentation schemes (2,3,5)—see mass chromatographic curves in Figures 2 and 4.

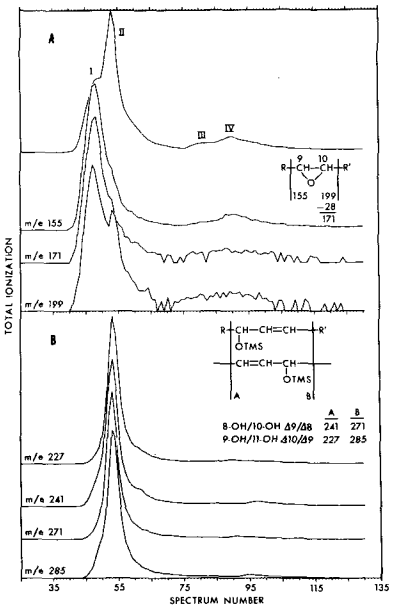

FIG. 2. Mass chromatography for the identification of: A. I = Methyl 9,10-epoxyoctadecanoate; B. II = Methyl 8-, 9-, 10-, 11-hydroxyoctadecenoate (OTMS); in $NaBH_4$ reduced-autoxidized methyl oleate (PV 1370).

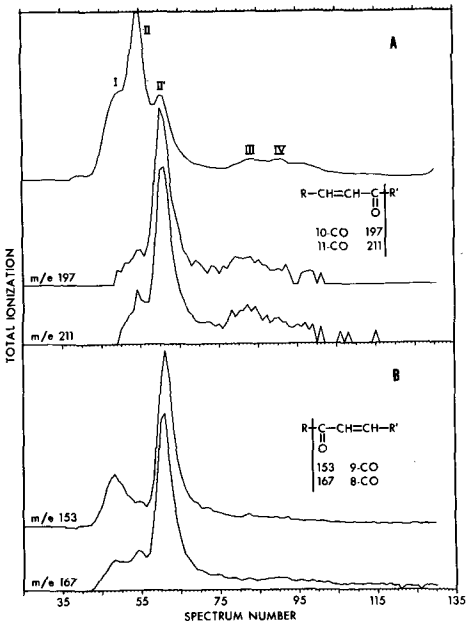

FIG. 3. Mass chromatography for the identification of II': A. Methyl 10-, 11-keto octadecenoate; B. Methyl 8-, 9-keto octadecenoate; in KI reduced-autoxidized methyl oleate.

formed with glass columns (193 x 0.4 cm) packed with 3% OV-101 on Gas-Chrom W 100/120 mesh (Applied Science, State College, PA), programming from 200 to 250 at 2 C/min.

Authentic 8-, 9-, 10-, and 11-hydroxyoctadecanoate were synthesized from the keto esters by literature methods (9) and reduced by $NaBH_4$. Other reference compounds included 9,10-epoxyoctadecanoate and 9,10-dihydroxyoctadecanoate prepared from methyl oleate (10).

RESULTS

Methyl oleate was treated with O_2 at different temperatures to reach different peroxide values. The autoxidized products were reduced with either KI or $NaBH_4$ to convert hydroperoxides into corresponding allylic alcohols. Under the conditions of the iodometric peroxide value determination, KI was not effective in completely reducing the hydroperoxides. The lipid extracted after KI reduction showed evidence of hydroperoxides and unsaturated ketones by thin layer chromatography (TLC). Therefore, the peroxide determination would appear to give erroneous results. $NaBH_4$ proved to be effective in completely reducing the hydroperoxides. However, with this reagent any allylic ketones would be reduced together with the hydroperoxides. Therefore, some samples

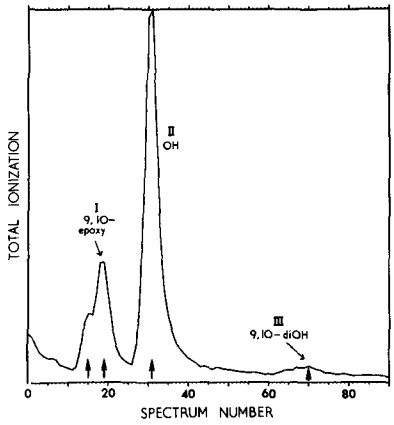

FIG. 4. GC-MS of silyl ethers of hydrogenated-autoxidized methyl oleate (PV 1370).

were divided and one part analyzed after KI reduction and the other after $NaBH_4$ reduction.

Autoxidized-reduced fatty esters were silylated; the resulting trimethylsilyl ethers of hydroxy esters have good GC properties and produce characteristic fragmentation by MS (2). Figure 1 shows gas chromatograms based on computer traces of total ionization determined by MS. The spectrum numbers correspond to full MS scan recorded every 12 sec. The chromatogram of the $NaBH_4$-reduced oxi-

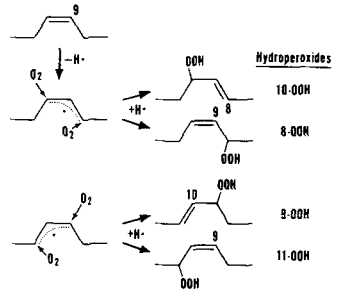

FIG. 5. Mass chromatography of hydrogenated-autoxidized methyl oleate (PV 1370) for the identification of: A. Methyl 8-OH octadecanoate (OTMS); B. 9-OH octadecanoate (OTMS); C. 10-OH octadecanoate (OTMS); D. 11-OH octadecanoate (OTMS).

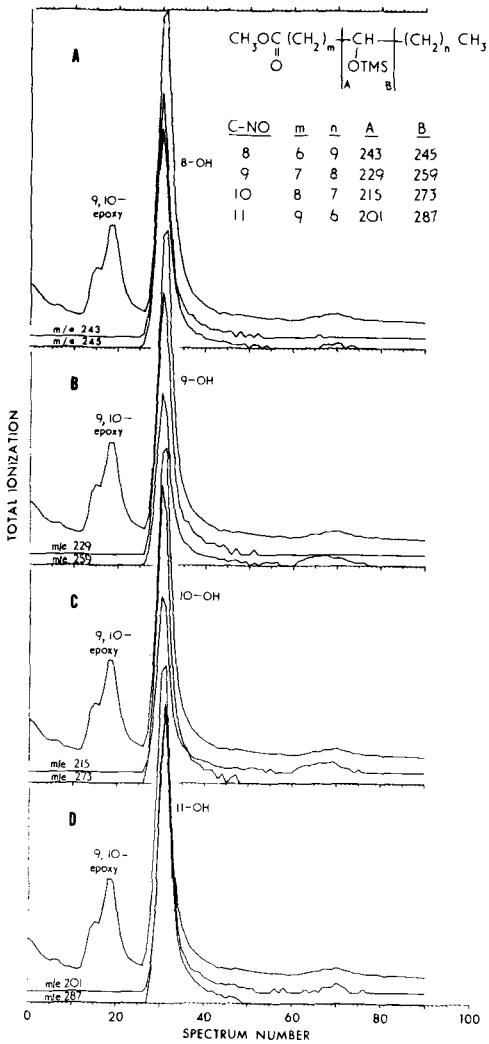

FIG. 6. Mechanism of oleate autoxidation.

dized oleate (Fig. 1A) showed a major unresolved peak due to a mixture of epoxyoctadecanoate (mainly 9,10-) (I) and the TMS derivatives of allylic alcohols from the 8-, 9-, 10-, and 11-oleate hydroperoxides (II) (Table I, part 1). Peak III was tentatively identified as due to isomeric dihydroxyoctadecenoates (TMS ethers) and peak IV to the corresponding saturated derivatives. The 9,10-dihydroxy isomers predominated in both peaks (Table I, part 1). The chromatogram of the KI-reduced oxidized oleate showed a smaller peak II (relative area: 40%, Fig. 1B) than the corresponding $NaBH_4$-reduced sample (relative area: 49%, Fig. 1A). In the KI-reduced sample, and additional small component appeared in the back side of peak II which was tentatively identified as due to unsaturated keto esters (peak II'). Evidently these enones are reduced to alcohols in the $NaBH_4$-treated samples.

Use of an MS-computer system (11) affords structural assignment of GC peaks due to incompletely resolved mixtures by computer plots of selected masses vs. retention times (spectrum number). By this method 9,10-epoxyoctadecanoate was identified by plotting characteristic masses 155, 171, and 199 and shown to be eluted in peak I. The TMS ethers of allylic 8-, 9-, 10-, and 11-hydroxy esters with characteristic masses 227, 241, 271, and 285 are eluted in peak II (Figs. 2A and 2B). The MS-computer system also provided evidence of 9,10-dihydroxyoctadecenoate (TMS ether) as the main component eluted under peak III (masses 257 and 259) and of 9,10-dihydroxyoctadecanoate (TMS ether) under peak IV (masses 215 and 259). In the KI-reduced oleate, evidence for allylic 8-, 9-, 10-, and 11-enones eluted under peak II' was obtained by the characteristic masses 197, 211, 153, and 167 (Figs. 3A and 3B).

After double bond hydrogenation, GC peaks due to isomeric epoxy- and hydroxyoctadecanoate (TMS ether) were well separated (Fig. 4). The MS resulting from individual scans in peaks I, II, and III were matched with the MS of reference compounds and identified in Table I (part 2). The evidence indicated that the main component 9,10-epoxyoctadecanoate was accompanied by small amounts of 8,9- and 10,11-epoxyoctadecanoate in the tail end of peak I. Peak II is due to the mixture of 8-, 9-, 10-, and 11-hydroxyoctadecanoate (TMS ethers) expected from oleate hydroperoxides. Peak III is attributed to a mixture consisting of 8,10-; 8,11-; 9,10-; and 9,11-dihydroxyoctadecanoate (TMS ethers) (Table I, part 2). These identifications are also supported by comparison of GC retention and MS data with those of

TABLE II

GC-MS Analysis of Synthetic Hydroxyoctadecanoate
Relative % Composition

Methods of analysis		8-OH Fragment A[a] Fragment B[a]	9-OH 243 245	10-OH 229 259	11-OH 215 273	201 287	Standard deviation, s
Mean relative % for 5 mixtures			25.40	20.94	23.92	29.74	
Computer summation	A + B		26.40	20.16	23.18	30.26	0.72
Peak spectrum	A + B		24.92	19.64	23.10	32.34	1.30
Peak spectrum	A		21.26	18.38	24.42	35.96	1.74

[a]See fragmentation scheme in Figure 5A.

TABLE III

GC-MS Analysis[a] of Isomeric Hydroxyoctadecanoate from Autoxidized Methyl Oleate

Peroxide value	Temperature, C	Relative percent 8-OH	9-OH	10-OH	11-OH
461	25	26.6	24.3	22.3	26.8
72	40	26.3	24.7	22.3	26.7
200	40	27.5	22.9	22.1	27.5
401	40	27.9	23.3	21.7	27.1
282	60	27.1	23.0	22.8	27.1
597	60	26.2	21.7	24.0	28.1
791	60	27.5	22.7	23.1	26.7
355	80	26.4	23.8	23.4	26.4
775	80	26.8	23.4	23.4	26.4
1232	80	26.2	23.9	23.9	26.0

[a]Based on computer summation of fragments A + B (see Table II).

reference dihydroxy esters.

The MS-computer system provided evidence of partial separation of the hydroxyoctadecanoate isomers under the GC conditions used (Fig. 5). Because of this separation, manual scanning of the peak due to hydroxyoctadecanoate may have caused errors in the quantitative analyses. These errors are apparently due to uneven measurement of the MS on only a portion, usually the apex, of the GC peak due to the mixture of hydroxyoctadecanoate isomers. In the quantitative analyses presented below, this problem was overcome by careful computer summation of total ions from all mass spectra obtained within the peak due to hydroxyoctadecanoate.

The quantitative GC-MS analysis was standardized by the use of synthetic samples of 8-, 9-, 10-, and 11-hydroxyoctadecanoate. Artificial mixtures of these synthetic hydroxy esters were analyzed by GC-MS based on three methods: (a) relative intensity of fragments A and B after computer summation of all the spectra within the GC peak due to hydroxyoctadecanoate (Fig. 5), (b) relative intensity of fragments A and B in the spectrum taken at the apex of the GC peak, and (c) relative intensity of fragment A in the spectrum taken at the apex of the GC peak. The results in Table II show that the most reliable quantitative method, with the lowest standard deviation of 0.72, was based on the computer summation of both fragments A and B within all the GC peak due to hydroxyoctadecanoate. Analyses based on fragment A give erroneously high values for the 11-hydroxy isomer and correspondingly low values for the 8-hydroxy isomers.

Samples of methyl oleate oxidized to different peroxide values and at different temperatures were analyzed by the computer summation approach (method a). The isomeric distribution summarized in Table III shows consistently higher relative concentrations of 8- and 11- than 9- and 10-hydroxy esters. The difference between these pairs of isomers varied at the PV range of 200-600 between 5.6% at 80 C and 10% at 40 C. The lowest difference between these pairs of isomers of 4.0% was shown by the most oxidized sample (PV 1528). This uneven distribution of isomers has important implications on the mechanism of autoxidation and indicates that, contrary to general belief (12), carbons -8, -9, -10, and -11 of oleate are not equivalent to oxygen attack.

TABLE IV

Literature on the Isomeric Hydroperoxides of Autoxidized Methyl Oletae

Reference	Autoxidation conditions	Basis of characterization	Isomeric distribution
13	35 C – UV	Beckman rearrangement of oximes from keto derivatives	10-OH > 11-OH > 8-OH > 9-OH
14	35 C	Infrared studies	9-OH = 10-OH
15	Room temperature	Cleavage of acetyl derivatives	8-OH = 9-OH = 10-OH = 11-OH
16	28 C	Cleavage of hydroxy derivatives	9-OH = 10-OH
17	Acidic MeOH	Cleavage of diMe acetal derivatives	8-OH > 11-OH > 9-OH > 10-OH
3	20 C		11-OH > 10-OH > 8-OH > 9-OH
	40 C	GC-MS of OTMS derivatives	11-OH > 9-OH > 10-OH > 8-OH
	80 C		8-OH > 9-OH > 10-OH > 11-OH

DISCUSSION

Different structural characterization schemes have been used to determine the isomeric distribution of oleate hydroperoxides, and the results of different workers are summarized in Table IV. Some of the characterization schemes are long, give low yields of products, and their quantitation has been questioned previously (12). The most recent work of Piretti et al. (3) is based on GC-MS analysis, but no synthetic hydroxy esters were used as references. Their quantitation was based on mass spectra taken at the apex of the chromatograhic peak and on the intensity of fragment A (See Fig. 5A), assuming that it is independent of the position of the TMS ether. When we applied this approach with our authentic samples of hydroxyoctadecanoates, it led to erroneous results (Table II). The partial separation of isomeric hydroxy esters by GC has been demonstrated (Fig. 5), and measurements based on MS taken at the apex of GC peaks can cause errors in the quantitative analyses. This problem was overcome by summation of all mass spectra within the appropriate peak by computer. Furthermore, reliable quantitation of the MS data was obtained with authentic hydroxy esters only if the intensity of both fragments A and B (Table II) were used in the calculations. This approach provides a direct and reliable method for the analysis of isomeric oleate hydroperoxides. In the analyses of different samples of oleate exposed to a wide range of autoxidation conditions, we found that the 8- and 11-hydroxy isomers were consistently higher than the 9- and 10-hydroxy isomers. The difference between these pairs of isomers was greatest when oleate was autoxidized at 40 C and smallest at 80 C and at high levels of autoxidation.

The most accepted mechanism for oleate autoxidation (12) involves hydrogen abstraction at the carbon-8 and -11 positions. The interaction between the unpaired electron on carbon 8- or carbon-11 and the π-electrons of the adjacent double bond produces two allylic radicals in with the electrons are delocalized over three carbon atoms (Fig. 6). These allylic hybrid radicals have two equivalent sights for oxygen attack: carbon-8 and carbon-10 on the one hand, carbon-9 and carbon-11 on the other. According to this scheme, the expected distribution of oleate hydroperoxides would include equal amounts of 8-, 9-, 10-, and 11-isomers. This does not, therefore, provide an adequate explanation for the uneven distribution of isomers found in the present study. Allylic hydroperoxides are also known to undergo allylic rearrangement (18), and this isomerization may influence the ultimate composition of oleate hydroperoxides.

Minor products tentatively identified by GC-MS in oxidized-reduced or hydrogenated oleate include allylic enones, epoxy-octadecanoates (8,9-, 9,10-, and 10,11-), dihydroxyoctadecenoates (8,9-, 9,10-, and 10,11-), and dihydroxyoctadecanoates (8,9-, 8,10-, 8,11-, 9,10-, 9,11-, and 10,11-). Among these products, those reported previously include the enones, 9,10-epoxyoctadecanoate, and 9,10-dihydroxyoctadecanoate (19-23). The allylic enones may be formed by dehydration of the hydroperoxides (19). Epoxy- and dihydroxy esters may come from either secondary reactions between oleate and the hydroperoxides or from the hydroperoxides themselves (24). In the first case the 9,10-isomers would be the only products. Our evidence for the other isomers indicates that the epoxy esters are also derived from the hydroperoxides. The vicinal-dihydroxy esters, in turn, may be derived from the isomeric epoxy esters (22,23). Finally, the 1,3-dihydroxy esters may come from dihydroperoxides (12).

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: II. Methyl Linoleate[1]

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ABSTRACT

The gas chromatography-mass spectrometry (GC-MS) approach developed in the preceding paper was applied for qualitative and quantitative investigations of autoxidation products of methyl linoleate. A GC-MS computer summation method was standardized with synthetic 9- and 13-hydroxyoctadecanoate. Equal amounts of 9- and 13-hydroperoxides were found in all samples of linoleate autoxidized at different temperatures and peroxide levels. The results are consistent with the classical free radical mechanism of autoxidation involving a pentadiene intermediate having equivalent sites for oxygen attack at carbon-9 and carbon-13. Minor oxygenated products of autoxidation indicated by GC-MS include keto dienes, epoxyhydroxy monoenes, di- and tri-hydroxy monoenes. These hydroxy compounds are presumed to be present in the form of hydroperoxides. The quantitative GC-MS method was found suitable for the analysis of autoxidized mixtures of oleate and linoleate. By this method, it is possible to determine the origin of the hydroperoxides formed in mixtures of these fatty esters.

INTRODUCTION

In the preceding paper (1), gas chromatography-mass spectrometry (GC-MS) was used for qualitative and quantitative investigations of autoxidized methyl oleate. In this paper, we report an extension of these studies to methyl linoleate.

MS and GC-MS have been used in a number of studies to help identify products from the reaction of lipoxygenase with linoleic acid and some of their enzymatic and nonenzymatic decomposition products (2-7). Apparently, only one report has appeared recently where GC-MS was used with autoxidized linoleate to characterize hydroperoxides and their thermal decomposition products (8). Partial separation of the 9- and 13-hydroxyoctadecanoate derivatives by GC permitted an estimate of their relative concentrations but no authentic compounds were used as references. We have shown in the preceding paper (1) that the application of GC-MS for quantitative analyses requires careful standardization with known synthetic compounds. In the present paper, a quantitative GC-MS computer summation method was so standardized with known mixtures of synthetic 9- and 13-hydroxyoctadecanoates. Although work has been published on the relative rates of oxidation of oleate, linoleate, and linolenate (9,10), no suitable method has been available for the analysis of the individual hydroperoxides formed in mixtures of these fatty esters. We used a direct GC-MS method to determine the isomeric hydroperoxide composition of autoxidized mixtures of oleate and linoleate. GC-MS provided also qualitative information on some of the secondary oxygenated products in autoxidized linoleate that may be precursors of off-flavors in fats.

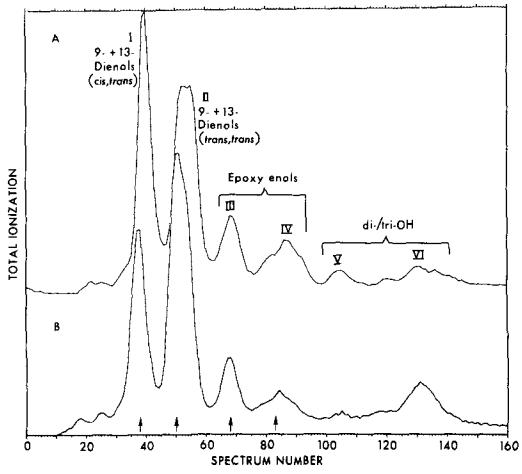

FIG. 1. GC-MS of silyl ethers of reduced-autoxidized methyl linoleate (PV 2128): computer traces of MS total ionization vs. spectrum numbers corresponding to full MS scan recorded every 12 sec. A. $NaBH_4$-reduced; B. KI-reduced.

[1]Presented at the AOCS Meeting, Chicago, September 1976.

TABLE I

Mass Spectral Data

Peak	Spectrum no.	Characteristic fragments m/e (rel. abundance)	Identification[a] (C-18 OTMS methyl esters)	References
Figure 1A[b] $NaBH_4$ reduced-oxidized linoleate				
I	40	225(100), 311(76.4)	9-OH-diene + 13-OH-diene	16
II	54	225(100), 311(90.0)	9-OH + 13-OH-diene	16
III	69	199(100) 285(88.4), 383(7.3) M - 15	11-OH-9,10-epoxy-ene + 11-OH-12,13-epoxy-ene	4-7
IV	87	173(100), 241(59.2) 259(68.6), 327(28.5)	9-OH-12,13-epoxy-ene + 13-OH-9,10-epoxy-ene	3,7
V	106 120	173(18.0), 259(16.8), 355(3.8) 173(56.7), 259(100), 355(17.1)	9,13-diOH-ene 9,13-diOH-ene	8
VI	131 136	173(89.8), 259(100), 301(9.8) 173(100), 259(91.8), 301(20.3)	9,12,13-triOH-ene + 9,10,13-triOH-ene	3,5,6,8
Figure 1B[b] KI reduced-oxidized linoleate				
III	68	151(100), 237(54.1), 308(74.0)	9-keto diene + 13-keto diene	5,7
Figure 3[c] Hydrogenated-oxidized linoleate				
I	15	155(87.0), 185(43.7), 281(27) M - 31 99(100), 241(30.6)	9-keto-ane 13-keto-ane	14
II	23	229(87.5), 259(94.5) 173(100), 315(59.1)	9-OH-ane 13-OH-ane	8
III	40	173(57.4), 301(25.2)[d] 259(70.2), 215(100)	11-OH-9,10-epoxy + 12,13-diOH-ane + 9,10-diOH-ane	3,4,7
IV	53	215(34.8), 259(64.0), 273(100) 173(100), 301(41.5)	9,10-/10,13-diOH-ane 12,13-diOH-ane	8
V	81	173(46.1), 213(59.8) 259(53.7), 299(62.5)	9,12,13-/9,10-13-triOH-ane	3,5,8

[a]See fragmentation schemes in Figure 2 and in preceding paper (1).

[b]m/e normalized from 100 to 400.

[c]m/e normalized from 0 to 400.

[d] 

EXPERIMENTAL PROCEDURES

Methyl linoleate was prepared from methyl esters of safflower oil and purified by counter double current distribution (11), silicic acid chromatography, and vacuum distillation. It analyzed 99.6% by GC (probable impurity, 0.4% conjugated diene).

The procedure for autoxidation, methods for peroxide values and GC, procedures for KI and $NaBH_4$ reduction, catalytic hydrogenation, silylation, and GC-MS were described in the previous paper (1). Authentic methyl 9- and 13-hydroxyoctadecanoate were prepared by $NaBH_4$ reduction of the corresponding keto derivatives synthesized by literature methods (12).

RESULTS

The GC-MS computer chromatogram of silyl ethers of oxidized-reduced samples of linoleate showed two major peaks due to the allylic 9- and 13-dienols from corresponding hydroperoxides (Fig. 1). Peak I was assigned to *cis,trans*-dienol and peak II to *trans,trans*-dienol on the following bases. With increasing level of oxidation, the relative area of peak II increased, and infrared (IR) analyses showed more intense bands at 990 (*trans,trans* + *cis,trans*) than at 955 cm^{-1} (*cis,trans*) (13). Also, the relative porportion of peak II increased when KI was the reducing agent (Fig. 1B). Apparently, somerization of *cis,trans*- to *trans,trans*-dienols

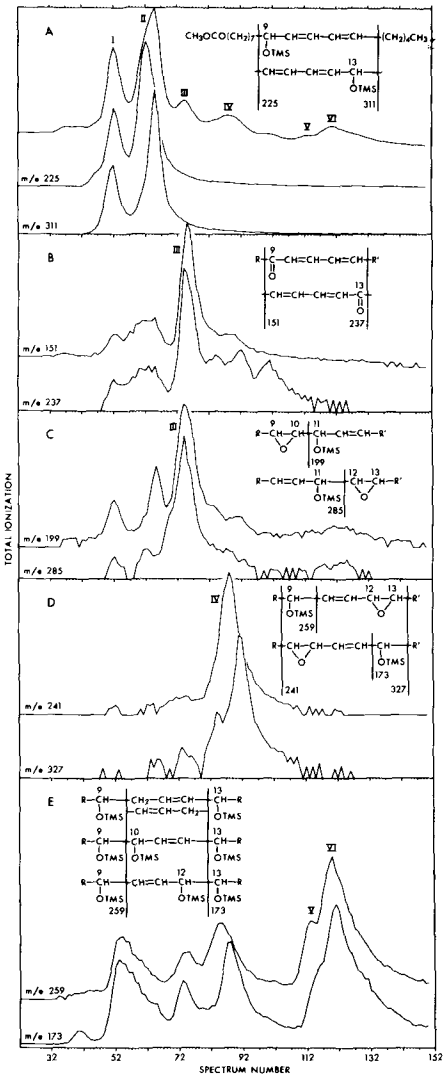

FIG. 2. Mass Chromatography of KI reduced-autoxidized methyl linoleate (PV 1403) as TMS ethers, for the identification of: A. I, II = methyl 9- and 13-hydroxyoctadecadienoate; B. III = methyl 9- and 13-keto octadecadienoate; C. III = methyl 11-hydroxy-9,10-epoxy-12-octadecenoate + methyl 11-hydroxy-12,13-epoxy -9- octadecenoate; D. IV = methyl 13-hydroxy-9,10-epoxy-11-octadecenoate + methyl 9-hydroxy-12,13-epoxy-10-oxtadecenoate; E. V, VI = methyl 9,13-dihydroxyoctadecenoate + methyl 9,12,13-/9,10,13-trihydroxyoctadecenoate.

was catalyzed by the I_2 produced from oxidized KI. The MS evidence indicates that epoxyenols are eluted from peaks III and IV, dihydroxy esters from peak V, and trihydroxy esters from peak VI (Table I). In the KI-reduced sample (Fig. 1B), components eluted from peak III show also MS evidence of small

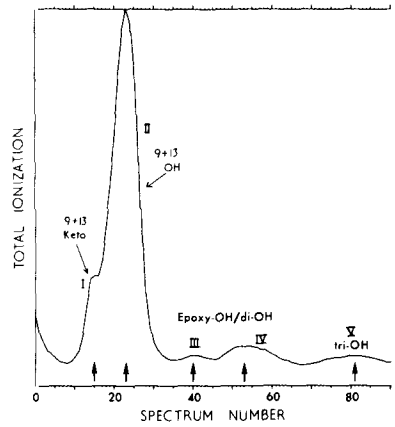

FIG. 3. GC-MS of silyl ethers of hydrogenated-autoxidized methyl linoleate (PV 2128).

TABLE II

GC-MS Analysis[a] of Synthetic Hydroxyoctadecanoate

Known mixtures	Relative percent 9-OH	13-OH
Mixture 1	48.7	51.3
Found	49.6	50.4
Mixture 2	44.9	55.1
Found	46.1	53.9
Mixture 3	77.5	22.5
Found	77.0	23.0
Mixture 4	50.7	49.3
Found	51.4	48.6
Mixture 5	81.0	19.0
Found	81.8	18.2
Mean relative %		
5 Mixtures	60.56	39.44
Found	61.18	38.82
St. deviation	0.92	

[a]Based on computer summation of masses 229 + 259 for 9-OH isomer and masses 173 + 315 for 13-OH isomer (1).

amounts of keto dienes. These components are reduced by $NaBH_4$ into the corresponding dienols. The identifications given in Table I are based on published fragmentation schemes (3-8,14-17) and confirmed below by mass chromatography and GC-MS analyses after catalytic hydrogenation of the autoxidized samples.

Mass chromatography (1) showed that the *cis,trans*-9-, and 13-dienols are not separated in peak I, and that the corresponding *trans,trans*-isomers are partially separated in peak II (Fig. 2A). Characteristic masses for keto dienes (m/e 151 and 237) and 11-hydroxy-9,10-/12,13-epoxy-enes (m/e 199 and 285) fall in peak III

(Fig. 2B, 2C), for 13-/9-hydroxy-9,10-/12,13-epoxy-enes (m/e 173, 241, 259, and 327) in peak IV (Fig. 2D, 2E), for 9,13-dihydroxyenes and 9,12,13-/9,10,13-trihydroxyenes (m/e 173 and 259) in peaks V and VI (Fig. 2E).

After double bond hydrogenation of the autoxidation products, the gas chromatogram shows evidence of 9- and 13-ketooctadecanoate (peak I), 9- and 13-hydroxyoctadecanoate (peak II), epoxyhydroxyoctadecanoate, dihydroxyoctadecanoate (peaks III-IV), and trihydroxyoctadecanoate (peak V) (Fig. 3, Table I). The diminished amount of epoxy esters indicates some reduction of the epoxy group during catalytic hydrogenation. There was no evidence of 11-hydroxyoctadecanoate (m/e 201 and 287) coming from peak II. These identifications were confirmed by mass chromatography. The evidence for dihydroxy esters with one hydroxy group on carbon-9 or -12 and the other on carbon-10 or -13 means that the corresponding monounsaturated dihydroxy esters were present before hydrogenation. Mass chromatography showed partial separation of the TMS ethers of 9- and 13-hydroxyoctadecanoate by GC. The 9-TMS ether isomers was eluted in the first half of peak II and the 13-TMS ether isomer in the second half. Because of this separation, quantitative determination of the 9- and 13-hydroxy esters requires careful summation of total ions for all mass spectra taken within peak II.

The quantitative GC-MS analysis was standardized with synthetic mixtures of 9- and 13-hydroxyoctadecanoate. The same computer summation method was used as that developed in the preceding paper for oleate (1). The results in Table II show that this computer summation method is quantitatively reliable, with a standard deviation of 0.92. Samples of methyl linoleate oxidized to different peroxide values and at different temperatures show a uniformly equal distribution of the 9- and 13-hydroxy esters (Table III). These results support the general belief (18) that carbons-9 and -13 of methyl linoleate are equivalent sites to oxygen attack.

The quantitative GC-MS method was also applied to the analysis of autoxidized mixtures of oleate and linoleate. Five mixtures were autoxidized at different levels, and the hydroxyoctadecanoate TMS derivatives were anal-

TABLE III

GC-MS Analysis[a] of Isomeric Hydroxyoctadecanoate in Autoxidized Methyl Linoleate

Peroxide value	Temp., °C	Relative percent 9-OH	13-OH
152	40	50.2	49.8
261	40	51.7	48.3
686	40	49.7	50.3
918	40	49.6	50.4
93	60	47.3	52.7
505	60	51.5	48.5
1403	60	49.0	51.0
1249	80	52.5	47.5

[a]Based on computer summation of masses 229 + 259 for 9-OH isomer and masses 173 + 315 for 13-OH isomer (1).

TABLE IV

GC-MS Analysis of Autoxidized Mixtures of Oleate:Linoleate (80 C)

Mixtures oleate:linoleate (Ol)	(Lo)	Peroxide value	Relative percent 8-OH	9-OH	10-OH	11-OH	13-OH	Origin[a] Ol	Lo
9:1		84	13.2	36.7	11.3	14.4	24.4	51.2	48.8
		152	13.3	33.4	11.7	16.2	25.4	49.2	50.8
		528	14.3	35.8	10.8	13.8	25.3	49.4	50.6
		1109	25.8	23.4	21.7	25.8	3.3	93.4	6.6
2:1		414	5.2	43.3	4.3	8.3	38.9	22.2	77.8
		995	5.5	42.9	5.8	11.1	34.7	30.6	69.4
		1047	9.5	40.3	7.0	9.1	34.1	31.8	68.2
1:1		106	3.1	46.9	1.8	3.2	45.0	10.0	90.0
		407	4.1	47.6	3.0	3.5	41.8	16.4	83.6
		1070	6.9	42.5	5.5	6.3	38.8	22.4	77.6
1:2		515	2.6	46.5	2.1	3.6	45.3	9.5	90.5
		1037	4.5	44.8	3.0	3.4	44.4	11.2	88.8
1:9		593	1.5	48.8	0.4	0.7	48.6	2.8	97.2
		936	0.3	49.2	1.2	1.2	48.1	3.8	96.2

[a]Assuming that amount of 9-OH = 13-OH in oxidized linoleate. Lo - hydroperoxides = 13-OH x 2; Ol - hydroperoxides = 8-OH + (9-OH - 13-OH) + 10-OH + 11-OH.

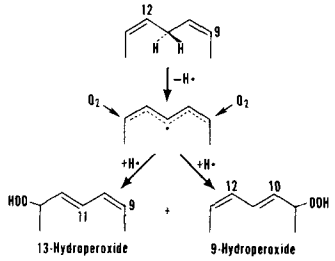

FIG. 4. Mechanism of linoleate autoxidation.

yzed quantitatively by GC-MS. With these mixtures, the 9-hydroxy ester comes from both oleate and linoleate hydroperoxides, whereas the 13-hydroxy ester comes only from linoleate hydroperoxides. Assuming that equal amounts of 9- and 13-hydroperoxides are formed from linoleate (as shown in Table III), it is possible to estimate the origin of the hydroperoxides in these autoxidized mixtures (Table IV). With the 1:1 mixture of these esters oxidized to peroxide values of 106, 407, and 1070, the total hydroperoxides originating from linoleate varied from 78 to 90%. With the 9:1 oleate-linoleate mixture oxidized to peroxide values of 84, 152, and 528, as much as 50% of the hydroperoxides formed come from linoleate. At the more advanced level of oxidation (PV 1109), as expected, oleate hydroperoxides become dominant. With 2:1 and 1:2 oleate-linoleate mixtures, linoleate hydroperoxices are again dominant and vary from 68 to 90% of the total. The quantitative GC-MS method thus appears suitable to determine the kind of hydroperoxide formed in autoxidized mixtures of oleate and linoleate.

DISCUSSION

Much work has been reported on the autoxidation of linoleic acid and related compounds, and it is generally agreed that equal amounts of 9- and 13-hydroperoxides are formed as initial products (2,8,18). In the analyses of different samples of linoleate autoxidized at different levels and temperatures, we also found equal amounts of the 9- and 13-hydroxy isomers. These results agree with the accepted mechanism for linoleate autoxidation (18) involving hydrogen abstraction at carbon-11 (Fig. 4). The interaction of the unpaired electron on carbon-11 and the π-electrons of the adjacent double bonds produce an allylic radical in which the electrons are delocalized over five carbon atoms. This pentadiene radical has two equivalent sites for O_2 attack: carbons-9 and -13, and the products expected by this scheme are equal amounts of the 9- and 13-isomers. Although it has been spectulated before that an 11-hydroperoxide may be formed from linoleate by autoxidation (19-22), our present studies show no evidence for it. However, there is now evidence in the literature that the 9- and 13-hydroperoxides of linoleate undergo facile interconversion (23).

Minor products indicated by GC-MS in reduced- or hydrogenated- oxidized linoleate include ketodienes, epoxyenols, di- and trihydroxyesters. Both heterolytic and homolytic mechanisms have been invoked for the nonenzymatic thermal or metal-catalyzed decomposition of linoleate hydroperoxides, usually in aqueous or alcoholic solutions (24). In the neat linoleate system used in this study, the homolytic removal of •OH from the diene hydroperoxides would likely lead to oxy radicals. These intermediates can then form either keto dienes by further abstraction of H• (7) or epoxyhydroxy esters by cyclization with an α double bond and addition of •OH on either end of an allyl 3-carbon system (24). The dihydroxy or trihydroxy esters may be formed either by 1,2- or 1,4-addition of •OH to the conjugated diene system of the 9- and 13-dienol intermediates (24), or by formation of dihydroperoxides (18) or even trihydroperoxides. The role of these oxygenated products as possible precursors of off-flavors in oxidized fats remains to be established.

It is known that the presence of linoleate greatly accelerates the autoxidation of oleate (9,25). In a recent kinetic study of oleate-linoleate mixtures, the rates of oxidation were dependent on oleate concentration (26). Different kinetics observed at different peroxide values were explained by the different propagation and termination rates of oleate and linoleate. In the present study, although we started with equal mixtures of oleate and linoleate, about 80% of the hydroperoxides formed at three peroxide levels originated from linoleate. Even when the mixture contained only 10% linoleate, 50% of the hydroperoxides formed below 9% oxidation came from the linoleate. These results reflect the greater ease of hydrogen abstraction from linoelate compared with that from oleate. Since rates of propagation and termination during autoxidation are greatly influenced by such factors as temperature, catalysts, antioxidants, and peroxide level, it is extremely difficult to predict the contribution of different fatty acids when present in mixtures as in natural fats. The GC-MS method was shown to be suitable for the determination of individual hydroperoxides formed in autoxidized mixtures of oleate and linoleate. This

approach will form the basis of a direct method to analyze the origin of hydroperoxides in autoxidized fats that include mixtures of these fatty acids.

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Determination of Lipid Conjugated Dienes with Tetracyanoethylene-^{14}C: Significance for Study of the Pathology of Lipid Peroxidation

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ABSTRACT

A method for quantitative analysis of conjugated diene unsaturation has been developed utilizing tetracyanoethylene-^{14}C (TCNE-^{14}C) in a Diels-Alder condensation. The amount of C^{14} found in the Diels-Alder adduct has been shown to be a measure of conjugated diene content. The method has proven successful in analysis of a variety of triglycerides, phospholipids, and peroxidized tissue lipids. In the course of this work, a method for removing the fatty acid substituents from phospholipids using lithium aluminum hydride was developed. TCNE-^{14}C analysis for conjugated dienes in rat liver microsomal lipids after dosing with CCl_4 or $BrCCl_3$ has provided conclusive evidence that the increase in ultraviolet absorption at 233 nm of these lipids is due to conjugated dienes.

INTRODUCTION

Diels-Alder condensations are 1,4-cyclo additions of conjugated dienes and a dienophile to form six membered rings. Forty years ago it was shown (1) that maleic anhydride could be used as the dienophile in a Diels-Alder condensation for an analytical determination of the conjugated double bond content of tung oil. Quantitative analysis depended on titrating unreacted maleic anhydride. This method requires a high temperature, a long reaction time, and relatively large amounts of lipid. We have extended the scope of this analytical technique by utilizing tetracyanoethylene (TCNE) as a dienophile. TCNE is a highly reactive dienophile (2). The reaction of TCNE with most dienes is quantitative, highly specific, and rapid, even at room temperature. The high reactivity of TCNE allows for exceptionally mild reaction conditions and a short reaction time. Diels-Alder active dienes can be determined by back-titrating excess TCNE with cyclopentadiene (3). However, tetracyanoethylene-^{14}C (TCNE-^{14}C) can be prepared from TCNE and KCN-^{14}C in an exchange procedure (4). TCNE-^{14}C and diene react in a 1:1 ratio. The specific activity of the TCNE-^{14}C can be determined with a high degree of precision. By determining the amount of label in the Diels-Alder adduct formed from reaction of TCNE-^{14}C and lipids containing conjugated dienes, one can obtain a direct measure of the conjugated diene content of the lipid sample. This method was used for quantitative analysis of the conjugated diene content in triglycerides, in phospholipids, and in lipids extracted either from rat liver microsomes peroxidized in vitro, or from liver microsomes of rats poisoned with CCl_4 or $BrCCl_3$. The significance of these studies from the point of view of the chemical pathology of certain types of toxic liver injury is discussed below.

EXPERIMENTAL PROCEDURES

Materials

The following compounds were used as received: lithium aluminum hydride (Ventron Corp., Danvers, MA), β-γ-dipalmitoyl-L-α-cephalin (Sigma Chemical Co., St. Louis, MO), β-γ-dipalmitoyl-DL-α-lecithin (Sigma Chemical Co.), tripalmitin (Eastman Organic Chemicals, Rochester, NY) and *trans*-2-*trans*-4-hexadiene (Aldrich Chemical Co., Milwaukee, WI), and castor oil (Fisher Scientific Co., Fairlawn, NJ). Tetracyanoethylene (Eastman Organic Chemicals) was recrystallized from chloroform-acetone, 3:1, and then sublimed twice under vacuum at 100 C to get white needle-like crystals, mp 199-200 C. 7,7,8,8-Tetracyanoquinodimethane (Eastman Organic Chemicals) was recrystallized twice from acetonitrile. Potassium cyanide^{14}C (New England Nuclear, Boston, MA) was diluted with cold potassium cyanide (1.4 g), dissolved in a minimum amount of water, and precipitated by addition of ethanol. Acetonitrile (Fisher Scientific Co.) was distilled from phosphorus pentoxide and stored under nitrogen. Tetrahydrofuran (Fisher Scientific Co.) was distilled and stored under nitrogen. Peroxide-free diethyl ether was prepared by washing the ether with a 5% ferrous sulfate solution (50 ml) which had

been acidified with sulfuric acid (0.5 ml). The ether was washed once with 5% sodium bicarbonate and once with water, and then dried by filtering through magnesium sulfate and anhydrous calcium sulfate. Commercial safflower oil was purified by dilution with petroleum ether and washing with 5% NaOH to remove antioxidants. The safflower oil was then washed with water, dried with anhydrous calcium sulfate, and the solvent removed under vacuum. Peroxidized safflower oil was prepared by bubbling pure oxygen into the well-stirred oil at 45 C for 3 weeks.

Methods

Preparation of tetracyanoethylene-^{14}C: The procedure we followed was an adaptation of the method of Webster et al. (4). A 3-necked 50-ml flask was equipped with a drying tube, a dropping funnel containing acetonitrile, and a gas inlet tube. Potassium cyanide-^{14}C (1.232 g; 18.94 millimoles) was added to the flask along with acetonitrile (20 ml). The well-stirred solution was then flushed with oxygen-free nitrogen for 10 min. TCNE (2.420 g; 18.94 millimoles) was dissolved in acetonitrile (25 ml) and degassed by passing oxygen-free nitrogen into the solution for 10 min. The TCNE solution was then added to the 3-nicked flask and stirred at 25 C for 2 hr under nitrogen. The solvent was then removed under reduced pressure and 7,7,8,8-tetracyanoquinodimethane (4.00 g; 19.6 millimoles) was added to the flask along with tetrahydrofuran (40 ml) and stirred for 1.5 hr. The solvent was removed under reduced pressure. The residue was then heated in a sublimer under reduced pressure at 100 C to give TCNE-^{14}C (0.705 g; yield 29%). The crude TCNE-^{14}C was recrystallized from chloroform-acetone, 4:1, and resublimed twice to give pure TCNE-^{14}C (226,000 dpm/mg). The yield of TCNE-^{14}C is variable (10-30%) and highly dependent on the correct stoichiometry of the starting reagents, purity of the reagents, and the complete exclusion of oxygen during the synthesis.

Preparation of methyl 9,11-octadecadienoate: Castor oil (30 g) was transmethylated using the method of Morgan and Hanahan (5). The resulting methylricinoleate was dehydrated using the procedure of Emken et al. (6) to yield a mixture of *trans,trans-*, *cis,trans-*, and *cis,cis*-conjugated methyl 9,11-octadecadienoate as well as other nonconjugated esters. Ultraviolet (UV) spectroscopic analysis showed 0.31 μmoles of conjugated diene per μmole of ester.

TCNE-^{14}C analysis for conjugated dienes in triglycerides: A sample of triglyceride (1-50 mg) or 2,4-hexadiene (0.04-4 mg) was dissolved in about 1 ml of chloroform. TCNE-^{14}C was added in 3 ml of chloroform. A convenient reaction vessel is a test tube fitted with a glass or plastic stopper. The quantity of TCNE-^{14}C added was ca. fivefold in excess of the estimated content of conjugated dienes in the triglyceride sample, on a molar basis. Specific activity of added TCNE-^{14}C was sufficient to yield ca. 10,000 dpm in the Diels-Alder adduct. If more than 9.5 mg of TCNE-^{14}C per 3 ml of chloroform was to be used, then just enough acetone was added to complete solubilization. The triglyceride and TCNE-^{14}C reagent were allowed to react at room temperature for 10 min. To remove the unreacted TCNE-^{14}C, the chloroform solution was washed four times with 5% sodium bicarbonate and once with water. The top aqueous layer was removed each time by aspiration. The solvent was then evaporated under a stream of nitrogen and the residue taken up in toluene. The radioactive Diels-Alder adduct in toluene was added to a toluene scintillant mixture of the following composition: toluene, 1 liter; 2,5-diphenyloxazone (PPO), 4 g; 1,4-bis-2,5-phenyloxazolyl benzene (POPOP), 50 mg. Radioactivity was monitored in a Model 2002 Tricarb Liquid Scintillation Spectrometer (Packard Instrument Co.).

Control runs were routinely carried out. These control runs contained all reagents except lipid. Any residual radioactivity due to TCNE-^{14}C not removed by the washing procedures was subtracted from experimental runs to yield net radioactivity of the Diels-Alder adduct. For lipids with a low content of conjugated dienes, the order of magnitude of this correction for unreacted TCNE-^{14}C was never greater than 15% of the radioactivity recovered in the Diels-Alder adduct. For most of the lipid samples analyzed, the correction was not greater than 2%.

TCNE-^{14}C analysis for conjugated dienes in phospholipids: Tetracyanoethylene reacts with the phosphate group of phospholipids (see below). Therefore, in order to quantitatively determine conjugated dienes in polyenoic fatty acid side chains of phospholipids, the fatty acids had to be cleaved from the phospholipids and subsequently isolated. This was done in two ways. One procedure involved use of lithium aluminum hydride to achieve reductive cleavage of fatty acid ester bonds. In this procedure, which to our knowledge is hitherto unpublished, the fatty acid ester

group is cleaved and the acyl group is converted to an alcohol. About 20 mg of lithium aluminum hydride was added to a well-stirred solution of phospholipid (10-100 mg) in 10 ml of anhydrous peroxide-free diethyl ether. At 10 min intervals, additional lithium aluminum hydride (about 20 mg) was added to the solution. Course of the cleavage reaction was followed by thin layer chromatography on silica gel with $CHCl_3$:CH_3OH:H_2O, 65:25:4, and was found to be complete in 30 min. Small pieces of ice were added to the ether solution to remove excess lithium aluminum hydride. After all excess lithium aluminum hydride was removed, the solution was acidified to a pH of 5 with 1 *N* hydrochloric acid. The ether layer was retained. The acidified aqueous layer was washed once with additional ether and the ether extracts combined. The ether extracts were washed once with water and evaporated to dryness using oxygen-free nitrogen. The recovery of the lipid residue was 97% of the theoretical value. The resulting lipid residue was analyzed using TCNE-^{14}C in the same procedure used for triglycerides.

In an alternate method, fatty acid residues of phospholipids were converted to methyl esters with 0.5 *N* NaOH in methanol by the method of Morgan et al. (5). Transmethylation of unsaturated phospholipids by this method followed by TCNE-^{14}C analysis of fatty acid methyl esters usually produced values for conjugated diene content 1-5% higher than values determined after lithium aluminum hydride cleavage indicating that a possible small amount of double bond isomerization had occurred during the transmethylation procedure.

TCNE-^{14}C analysis for conjugated dienes in peroxidized microsomal lipids: Rat liver (2.5 g) was homogenized in ice-cold saline-phosphate buffer (0.155 *M* NaCl and 0.05 *M* Na_2HPO_4, adjusted to pH 6.5). The homogenate was centrifuged at 5000 rpm for 12 min at 4 C in an SS-34 rotor of the Sorval RC2 B centrifuge. The supernatant fraction was removed. Additional saline-phosphate buffer was added to the supernatant fraction (which contains the microsome fraction and soluble cell constituents) to bring the final volume to 50 ml of saline-phosphate buffer for the extract derived from 1 g of liver. Saline-phosphate extracts of the liver prepared in this way were incubated in air for 1 hr with shaking in a water bath held at 38 C. For control incubations, sufficient ethylenediaminetetracetate (at least 0.0004 *M*) was added to completely prevent any lipid peroxidation. For the experimental incubations, in the absence of EDTA, the constituent lipids undergo peroxidative decomposition. Immediately after incubation, EDTA was added to the experiments to the same final concentrations as in the controls. The resultant mixtures were centrifuged at 17,500 rpm for 60 min (SS-34 rotor, Sorval RC2-B centrifuge). The supernatant fraction was discarded. The microsomal pellet was homogenized with methanol (10 ml) and transferred to a 40 ml conical centrifuge tube. Chloroform (20 ml) was added and the solution was shaken gently for 10 min. The chloroform-methanol solution was filtered through glass wool to remove undissolved protein. Water (10 ml) was added and the mixture shaken for 5 min and then cooled on ice. The mixture was then centrifuged at 1500 rpm for 10 min and the water layer and protein interface discarded. At this point, an aliquot of the chloroform layer (2 ml) was taken and analyzed spectrophotometrically for conjugated dienes by the method of Recknagel and Ghoshal (7) and for total lipid by the method of Chiang et al. (8). The remaining chloroform solution was evaporated using a stream of oxygen-free nitrogen and analyzed with TCNE-^{14}C by procedures developed for phospholipids. The lithium aluminum hydride method was used to reductively cleave fatty acid ester bonds.

In vivo lipid peroxidation induced by CCl_4 or $BrCCl_3$: Sprague-Dawley rats (Zivic- Miller Laboratories Inc., Allison Park, PA) with a body weight range of 180-220 g, were fasted overnight. Under light ether anesthesia, the rats were given $BrCCl_3$ (100 μl per 100 g body weight) or CCl_4 (500 μl per 100 g body weight) by intragastric administration. The toxigenic haloalkanes were administered in a small volume of mineral oil. Control rats received only mineral oil. After 90 min, the rats were killed and liver microsomal lipids were isolated as indicated above, except that the saline-phosphate buffer medium contained added EDTA (to prevent any further lipid peroxidation) and the microsomes, isolated by differential centrifugation, were kept ice-cold and processed for lipid extraction immediately after being harvested. The extracted microsomal lipids were analyzed for conjugated dienes with TCNE–^{14}C by procedures developed for phospholipids. Fatty acid ester bonds were cleaved reductively with lithium aluminum hydride.

Estimation of molar content of conjugated dienes by UV spectroscopy: Lipid conjugated dienes absorb intensely in the 233 nm region. The molar absorptivity varies depending on

structural isomerism, chain length, nearby functional groups, and nature of the solvent. Since the chemical identity of lipid conjugated dienes appearing in peroxidized lipids of biological origin is unknown, we used an extinction coefficient of 30,000 liter per mole per cm (9) as a working average. UV absorption of lipids was always measured at a lipid concentration of ca. 1 mg per ml of cyclohexane, and then rationalized to a common basis of exactly 1 mg per ml, after determination of the exact lipid content according to Chiang et al. (8).

RESULTS

Analysis for Conjugated Double Bonds

A pure sample of accurately weighed *trans*-2-*trans*-4-hexadiene was analyzed by TCNE-^{14}C adduct formation. The analysis proved to be consistent with the formation of 1 μmole of conjugated diene adduct per μmole hexadiene (1 ± 0.04) and was linear over the range 0.04 to 4 mg of 2,4-hexadiene (Fig. 1). These results conclusively show that the TCNE-^{14}C analysis is capable of accurately determining the conjugated double bond content of an alkene.

An experiment was designed to determine the effect of the configuration about the double bond (i.e., *cis-trans* isomerization) on TCNE-^{14}C adduct formation. A synthetic mixture of conjugated isomers, predominately methyl 9,11-octadecadienoate, was prepared by dehydrating methyl ricinoleate obtained from castor oil, according to the method of Emken et al. (6). This synthetic mixture contained 31% conjugated diene as measured by UV analysis (Table I). TCNE-^{14}C analysis showed a conjugated diene content of 25%. This discrepancy could be due to our choice of 30,000 as an average molar extinction coefficient for lipid conjugated dienes. Alternately, the discrepancy could be due to the presence of the *cis-cis* isomer. The inability, for steric reasons, of the *cis-cis* isomer to assume the cisoid (i.e., *s-cis*) conformation needed to effect Diels-Alder adduct formation would preclude reaction with TCNE-^{14}C. Emken's (6) analysis of a similarly prepared mixture of isomers of methyl 9,11-octadeca-

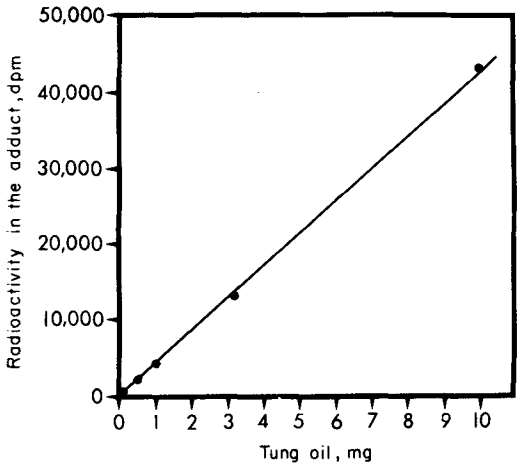

FIG. 1. Adduct of TCNE-^{14}C and 2,4-hexadiene as a function of μmoles 2,4-hexadiene analyzed. Specific activity of the TCNE-^{14}C used for the above assay was 4380 dpm/μm.

TABLE I

TCNE-^{14}C Analysis for Conjugated Double Bonds in Triglycerides

	Methyl 9,11-octadecadienoate	Peroxidized safflower oil	Safflower oil	Tripalmitin
Number of samples analyzed	2	4	4	4
dpm/mg[a]	1528	450 ± 20	29.4 ± 2	1.55 ± 0.09
Conjugated diene, micromoles per mg of lipid (TCNE-^{14}C)[b]	0.88	0.26	0.017	0.0009
Conjugated diene, micromoles per mg of lipid (U-V)	1.06	0.39	0.031	≤0.001
dpm/mg[c] after $LiAlH_4$ cleavage	---	427 ± 25	27 ± 2	---
dpm/mg[d] after transmethylation	---	480 ± 25	30 ± 2	---

[a]dpm in the Diels-Alder adduct per mg of lipid analyzed, ± standard deviation.

[b]Micromoles of conjugated dienes per mg of lipid, based on specific activity of the TCNE-^{14}C equal to 1730 dpm/μmole.

[c]dpm in the fatty alcohols derived by $LiAlH_4$ cleavage, per mg of initial lipid.

[d]dpm in the fatty acid methyl ester derived after transmethylation, per mg of initial lipid.

TABLE II

Quantitative Determination of Conjugated Dienes in Lipids Derived from Rat Liver Microsomes Peroxidized In Vitro

Line number	Source of lipid	Radioactivity dpm/mg lipid[a]	Conjugated dienes μmoles/mg lipid[b]	Absorption 233 nm[c]
1	Nonperoxidized microsomes	180	0.017	0.290
2	Peroxidized microsomes	780	0.074	2.10
	Conjugated diene content of peroxidized lipid (minus control value)		0.057 μmoles per mg lipid[d]	0.061 μmoles per mg lipid[e]

[a]dpm in the Diels-Alder adducts formed in the fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids.

[b]Micromoles of conjugated dienes in fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids, based on a specific activity of 10,500 dpm per micromole of TCNE-^{14}C.

[c]Absorption at 233 nm, rationalized to a concentration of 1 mg lipid per ml of cyclohexane.

[d]Line 2 minus line 1.

[e]Calculated from the difference in absorption (line 2 minus line 1) and assuming a molar absorptivity of 30,000 liters x $mole^{-1}$ x cm^{-1}.

dienoate revealed about 20% of the *cis-cis* isomer.

As expected, the saturated triglyceride tripalmitin was unreactive in the TCNE-^{14}C analysis (Table I). Although safflower oil is highly unsaturated, it contains only isolated (i.e., nonconjugated) double bonds. This oil was essentially unreactive under conditions of the analysis. However, as expected, peroxidized safflower oil reacted in the analysis (Table I). The conjugated diene content of peroxidized and nonperoxidized safflower oils estimated with TCNE-^{14}C was compared with values determined from an independent UV absorption analysis of the same oils (Table I). The values agreed well, considering the relatively low level of conjugated dienes in these safflower oil samples, and the uncertainty regarding the molar extinction coefficient for 233 nm absorption in peroxidized lipids.

Reaction of Saturated Phospholipids with TCNE-^{14}C

If TCNE reacted only with conjugated diene configurations, then saturated phospholipids should be inert in the reaction with TCNE-^{14}C. However, we found that 0.78 μmoles of TCNE-^{14}C reacted per μmole of β-γ-dipalmitoyl-L-α-cephalin, and that 0.92 μmoles TCNE-^{14}C reacted per μmole of β-γ-dipalmitoyl-DL-α-lecithin. Condensation of TCNE-^{14}C with the phosphate group is surmised; however, the nature of the stable reaction products was not further studied. The fatty acid side chains of both of these saturated phospholipids were transmethylated to fatty acid methyl esters. These methyl esters were completely inert in the TCNE-^{14}C analysis. These findings dictated that TCNE-^{14}C analysis of phospholipids for possible conjugated diene content would have to be preceded by cleavage of fatty acid ester bonds and subsequent isolation of fatty acids (or their residues) in a form amenable to reaction with TCNE-^{14}C. We used either the reductive lithium aluminum hydride procedure (see Methods) or transmethylation (5). Lithium aluminum hydride is inert to olefinic groups and rapidly reduces organic peroxides to nonperoxidic products, thus preventing any possible autocatalytic formation of lipid hydroperoxides and concomitant double bond isomerization during the reductive fatty acid ester cleavage reaction. TCNE-^{14}C analysis of long chain fatty alcohols derived from safflower oil showed that no double bond isomerization to conjugated dienes had occurred during lithium aluminum hydride cleavage (Table I).

Analysis for Conjugated Double Bonds in Peroxidized Tissue Lipids – In Vitro Lipid Peroxidation

Double bond isomerization along the fatty acid side chains of membrane lipids to produce conjugated double bond configurations is generally thought to occur during peroxidative cellular injury. The presence of conjugated double bonds is frequently determined by measuring absorbance of UV light at 233 nm. To establish firmly that the UV absorption is due to conjugated dienes, we used the TCNE-^{14}C reagent for detection and quantitative analysis of conjugated dienes in lipids extracted from rat liver microsomes

TABLE III

Quantitative Determination of Conjugated Dienes in Liver Microsomal Lipids of Rats Given CCl_4

Line number	Source of microsomal lipid	Radioactivity dpm/mg lipid[a]	Conjugated dienes μmoles/mg lipid[b]	Absorption 233 nm[c]
1	Control rats (6)	160 ± 13	0.015	0.280 ± .015
2	CCl_4 poisoned rats (6)	280 ± 14	0.027	0.610 ± .035
	Conjugated diene content of peroxidized lipid (CCl_4 poisoned minus control)		0.012 μmoles per mg lipid[d]	0.011 μmoles per mg lipid[e]

[a]dpm in the Diels-Alder adducts formed in the fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids, ± standard deviation.

[b]Micromoles of conjugated dienes in fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids, based on a specific activity of 10,500 dpm per micromole of TCNE-^{14}C.

[c]Absorption at 233 nm, rationalized to a concentration of 1 mg lipid per ml of cyclohexane; ± standard deviation.

[d]Line 2 minus line 1.

[e]Calculated from the difference in absorption (line 2 minus line 1) and assuming a molar absorptivity of 30,000 liters x mole^{-1} x cm^{-1}.

TABLE IV

Quantitative Determinationof Conjugated Dienes in Liver Microsomal Lipids of Rats Given $BrCCl_3$

Line number	Source of microsomal lipid	Radioactivity dpm/mg lipid[a]	Conjugated dienes μmoles/mg lipid[b]	Absorption 233 nm[c]
1	Control rats (6)	160 ± 13	0.015	0.280 ± .015
2	$BrCCl_3$ poisoned rats (4)	420 ± 20	0.040	0.890 ± .105
	Conjugated diene content of peroxidized lipid ($BrCCl_3$ poisoned minus controls)		0.025 μmole per mg lipid[d]	0.020 μmoles per mg lipid[e]

[a]dpm in the Diels-Alder adducts formed in the fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids, ± standard deviation.

[b]Micromoles of conjugated dienes in fatty alcohols derived by $LiAlH_4$ cleavage of 1 mg of microsomal lipids, based on a specific activity of 10,500 dpm per micromole of TCNE-^{14}C.

[c]Absorption at 233 nm, rationalized to a concentration of 1 mg lipid per ml of cyclohexane; ± standard deviation.

[d]Line 2 minus line 1.

[e]Calculated from the difference in absorption (line 2 minus line 1) and assuming a molar absorptivity of 30,000 liters x mole^{-1} x cm^{-1}.

peroxidized in vitro. The microsome fraction was obtained by differential centrifugation of normal rat liver homogenates (see Methods). Aliquots were allowed to peroxidize. In control incubations, peroxidation was prevented by addition of EDTA. Microsomal lipids were subsequently isolated, cleaved with lithium aluminum hydride, and analyzed for conjugated diene content with the TCNE-^{14}C reagent. Samples of extracted lipid were also analyzed for conjugated diene content by UV absorbance at 233 nm. The data (Table II) show clearly that the increase in UV absorbance is indeed due to the presence of conjugated dienes, as revealed by TCNE-^{14}C analysis. For these lipid samples, the TCNE-^{14}C method was about equally sensitive as the UV spectroscopic method.

Analysis for Conjugated Double Bonds in Liver Microsomal Lipids of Rats Fed CCl_4 or $BrCCl_3$

An important question to be answered was could these methods be used to conclusively demonstrate the presence of conjugated dienes in microsomal lipids isolated from livers of rats fed CCl_4 or $BrCCl_3$. The convictions that CCl_4 (7,10-12) and $BrCCl_3$ (13) initiate peroxidative decomposition of lipids in the liver cell endoplasmic reticulum of rats fed

these hepatotoxic agents rests heavily on the appearance of conjugated dienes in these lipid extracts, as revealed by UV spectroscopy. Experiments were carried out with rats given either CCl_4 or $BrCCl_3$. The results conclusively show that CCl_4 (Table III) and $BrCCl_3$ (Table IV) initiate double bond isomerization in lipids of the liver cell endoplasmic reticulum. The values obtained from the TCNE-^{14}C analysis are closely the same as the values obtained through UV light measurement.

DISCUSSION

The results reported in this communication are first of all significant from an analytical point of view. A new method has been developed for the detection and quantitative analysis of lipid conjugated dienes. This method utilizes TCNE-^{14}C in a Diels-Alder condensation. Although analysis for lipid conjugated dienes by UV spectroscopy is considerably faster and simpler, it is nonspecific. The method presented here, which depends on TCNE-^{14}C analysis, has the advantage of being chemically more rigorous than the UV method. However, it should be recognized that conjugated trienes, conjugated quatrenes, etc., will undergo Diels-Alder condensations with suitable dienophiles. For example, TCNE-^{14}C forms a 1:1 adduct with the conjugated trienes contained in tung oil (R.O. Recknagel and R.L. Waller, unpublished). Since lipids of biological origin, and especially of animal origin, rarely contain conjugated systems of more than two double bonds, the possible interference of higher order conjugated systems is unlikely and can be easily confirmed by UV analysis.

Apart from their intrinsic analytical interest, the results reported in this communication are significant from the point of view of the chemical pathology of toxic cellular injury. It is now recognized that peroxidative decomposition of the lipids of biological membranes is a very destructive process. This process is involved in a variety of pathological manifestations, usually but not necessarily of toxigenic origin. For example, the liver cell injury produced by CCl_4 and $BrCCl_3$ is due to peroxidative decomposition of lipids in the membranous component of liver cell endoplasmic reticulum (11,12). Lipid peroxidation results in mitochondrial swelling and disintegration and in disintegration of lysosomes. When lipids in the membranes of red blood cells undergo peroxidation, the red cells hemolyze. Lipid peroxidation has been invoked as a possible mechanism in the clinically important problem of oxygen toxicity (14). Lung damage induced by ozone (15,16) and by nitrogen dioxide (17) and seizures of central nervous system of origin induced by oxygen under high pressure (18) have been ascribed to lipid peroxidation. Peroxidative decomposition of lipids may be involved in cell damage caused by ionizing radiations or by vitamin E deficiency. This list of examples illustrates the variety of chemical and physical agents which are either known to cause or are suspected of being able to cause pathological changes in living cells by inducing peroxidative decomposition of membrane lipids. From an experimental point of view, a central problem in this area is to be able to determine whether particular end-stage pathological manifestations (i.e., epiphenomena such as necrosis, structural deformities, fatty degeneration, etc.) have or have not emerged as a result of lipid peroxidation. In other words, a central analytical problem is to determine for any given case whether the hypothetical sequence: initial events → lipid peroxidation → end-stage pathological change corresponds to reality. At the level of whole animal studies, this turns out to be a vexing problem. Due to the instability of organic peroxides (19,20), analytical methods for direct determination of lipid hydroperoxides cannot be relied on for quantitating lipoperoxidation in vivo. Lipid peroxidation can be detected in vitro by the thiobarbituric acid reaction (21) for malonaldehyde. In the case of carbon tetrachloride poisoning, early efforts to detect lipid peroxidation in vivo by assay for malonaldehyde were unsuccessful (22). It was subsequently shown (23) that malonaldehyde is metabolized over mitochondrial pathways in rat liver. This observation forced the realization (23) that failure to detect malonaldehyde in vivo could not be taken as evidence that lipoperoxidation had not occurred. A new approach to the problem (7,10) made use of the fact that when lipids containing polyenoic fatty acids undergo peroxidative decomposition, there is a shift of double bonds leading to appearance of conjugated dienes with intense absorption at 233 nm (9,21,24). When this method of analysis was applied to lipids extracted from the microsome fraction obtained by differential centrifugation of homogenates of CCl_4-poisoned rats, an increase in absorption in the 230-235 nm region was observed in comparison with microsomal lipids from control rats. The latter exhibit only smooth end-absorption as the wavelength is decreased toward 220 nm. The difference spectrum revealed an absorption peak near

235 nm. This method has since been widely used in study of liver injury induced by CCl_4 (25-28), $BrCCl_3$ (13), 1,1,2,2-tetrachloroethane (27), ethylene dibromide (28), ethanol (29), and other hepatotoxic agents. Data emerging from application of the method have served as the basis for considerable speculation (12) on the mode of action of these and other hepatotoxic agents. However, to our knowledge, rigorous evidence has not been presented to support the opinion that the increase in absorption is indeed due to appearance of conjugated dienes or that such conjugated dienes are present in the fatty acid side chains (or their residues) of extracted membrane lipids. Data presented in this communication demonstrate rigorously that the increase in absorption in the 230-235 nm region observed in rat liver microsomal lipids after CCl_4 or $BrCCl_3$ administration is due to appearance of conjugated dienes. This work permits the important conclusion that the fast and relatively simple method of UV spectroscopic scanning of extracted lipids, as applied according to Recknagel and Ghoshal (7), is a reliable method for detection of double bond isomerization in the fatty acid side chains of membrane lipids in vivo.

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Lithocholic Acid in Human Liver: Identification of ϵ-Lithocholyl Lysine in Tissue Protein

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ABSTRACT

Human liver had been shown to contain two forms of lithocholic acid. One form is extractable by 95% ethanol-ammonia, 1000:1, v/v (soluble lithocholate, SL) and the other form is firmly bound to the tissue residue. The latter, tissue-bound lithocholic acid (TBL), can be enzymatically released by means of the specific clostridial peptide bond hydrolase, cholylglycine hydrolase (cholanoyl amino acid hydrolase, EC no. 3.5). Solvolytic procedures for the analysis of hepatic lithocholic acid sulfate revealed that almost all of the TBL was non sulfated, while in the SL fraction there was an apparent preponderance of the sulfated form. Cholylglycine hydrolase liberates free labeled lithocholic acid from synthetic [24-^{14}C] lithocholyl-bovine serum albumin and from [24-^{14}C] lithocholyl polylysine. By analogy, the enzyme releases lithocholic acid from tissue protein in which the bile acid is conjugated through amino groups of basic side chains. Hydrolysis of [24-^{14}C] lithocholyl polylysine with 6N HCl yielded ϵ-[24-^{14}C] lithocholyl lysine, which was chromatographically similar to the product obtained by acid hydrolysis of TBL. Chromatographic, infrared, and mass spectroscopic studies with synthetic N-α-lithocholyl lysine, N-ϵ-lithocholyl lysine, and N-α-ϵ-Bis lithocholyl lysine showed the hydrolytic product from TBL to be N-ϵ-lithocholyl lysine. Since monohydroxy bile acids, such as lithocholic acid, are known to show unusual cytotoxic properties, the identification of TBL in human liver poses important questions regarding the role of lithocholate in liver injury.

INTRODUCTION

The naturally occurring primary bile acids, cholic and chenodeoxycholic acids, constitute an important class of end products of cholesterol metabolism (1). These bile acids are normally secreted into the bile as water-soluble conjugates of glycine or taurine. The ability of the liver to synthesize water-soluble bile acid conjugates, selectively, with the amino acids, glycine and taurine, is a normal property of hepatic cells. However, there are instances where hepatic and extrahepatic factors either alter the relative proportions of the glycine or taurine conjugates or induce the formation of atypical conjugates with other basic amino acids such as ornithine and arginine (2-6).

During the course of our studies on the bile acid composition of liver biopsies from patients who have had the intestinal bypass procedure for obesity (7), we found a small proportion of lithocholic acid firmly bound to tissue protein in a form nonextractable by the usual solvent system (95% ethanol-ammonia, 1000:1, v/v) described by us earlier (8). In this paper, we present evidence to show that some lithocholic acid occurs conjugated to protein via the ϵ-amino group of lysine and that this form of bound lithocholic acid can be released from hepatic tissues, using the specific clostridial peptide bond hydrolase, cholanoyl amino acid hydrolase (9, 10).

MATERIALS AND METHODS

Chemicals and Reagents

All solvents, reagent grade, were redistilled before use. Gas Chrom P and the liquid phases for gas liquid chromatography (GLC) were purchased from Applied Science Laboratories, State College, PA or from Supelco, Bellefonte, PA. N-α-carbobenzyloxy-L-lysine, N-ϵ-carbobenzyloxy-L-lysine, L-lysine monohydrochloride, and polylysine HBr (MW 5000) were purchased from Sigma Chemical Co., St. Louis, MO.

Cholanoyl amino acid hydrolase (cholylglycine hydrolase, Schwarz-Mann, Orangeburg, NY) used for the enzymatic hydrolysis of bile salt conjugates was prepared from cultures of *Clostridium perfringens* (ATCC-19574) as described earlier (9,10). [24-^{14}C] lithocholic acid (5.37 mCi/mM) was obtained from Mallinckrodt Jersey City, NJ. The coupling

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agent, 1-cylohexyl-3-3(2-morpholinoethyl) carbodiimide, metho-p-toluenesulfonate (carbodiimide) was purchased from Aldrich Chemical Co., Milwaukee, WI.

Extraction and Quantitation of Soluble and Tissue-Bound Lithocholic Acid (TBL)

Human liver, (50-100 mg) obtained from autopsy specimens, was homogenized with 10 ml of a mixture of 95% ethanol-ammonia (1000:1, v/v) using a Polytron tissue disintegrator. The homogenate was heated in a water bath for 10 min at 95 C and then centrifuged for 10 min at 5000 x G in a Sorvall RC 2B refrigerated centrifuge, using an SS-34 rotor. This extration procedure was repeated on the residue, the supernatants combined, evaporated under reduced pressure in a rotary evaporator, and set aside for analysis of soluble bile acids (lithocholic acid) by GLC. The residual pellet remaining after the extraction was dissolved in 0.5-1.0 ml of 1.0 M NaOH by warming in a hot water bath at 80 C and then set aside for the analysis of bound lithocholic acid.

The residue obtained by evaporation of the combined ethanol-ammonia extracts was dissolved in 0.5 ml of ethanol and then diluted with 2.0 ml of distilled water. After adjusting the pH to 5.8, the bile acid conjugates in the solution were enzymatically hydrolyzed with cholylglycine hydrolase in the presence of 0.1 M acetate buffer, pH 5.8, disodium EDTA, and β-mercaptoethanol for 2 hr at 37 C (10, 11). The hydrolysate was acidified to pH 1.0 with 6 N HCl, extracted four times with equal volumes of ethyl acetate, dried with anhydrous sodium sulfate, and then evaporated to dryness. The residue was dissolved in 110 ml of methanolic HCl (methanol saturated with anhydrous HCl), and bile acid methylation was allowed to proceed over night at room temperature. The solvent was evaporated under a stream of nitrogen, and the bile acid methyl esters, dissolved in a small volume of methanol, were subjected to thin layer chromatography (TLC) on Silica Gel G using benzene acetone (95:5) as the developing solvent (12). Methyl lithocholate run on a reference channel was visualized by exposure to iodine vapors, and zones corresponding to this standard were scraped and extracted with methanol-acetone (80:20). This extract was evaporated to dryness and methyl lithocholate was converted to its trifluoroacetate with 0.05-0.1 ml of trifluoracetic anhydride and subjected to GLC (11-13).

The tissue residue that had been dissolved in 1.0 M NaOH was diluted with an equal volume of water, adjusted to pH 5.8, and hydrolyzed with cholylglycine hydrolase as described in the previous paragraph (10,11). Under these conditions, the release of bound lithocholic acid was completed in 2 hr at 37 C. Lithocholic acid was isolated and determined by GLC in the form of its methyl ester trifluoroacetate as described in the previous paragraph (11-13).

Preparation of [24-^{14}C] Lithocholyl Polylysine and ϵ-[24-^{14}C] Lithocholyl Lysine

Polylysine (5.0 mg), MW 5000, was dissolved in 3.0 ml of 0.1 M borate buffer, pH 9.2, and the solution was allowed to react with [24-^{14}C] lithocholic acid (40 μCi in 0.3 ml ethanol), non radioactive lithocholic acid (2.0 mg in 0.4 ml ethanol) and carbodiimide (15 mg in 2.0 ml of 0.1 M borate buffer, pH 9.2). The mixture was stirred overnight and then centrifuged at 2000 rpm for 5 min. The precipitate, which contained conjugated [24-^{14}C] lithocholyl polylysine, was resuspened in the original volume of borate buffer and recentrifuged. The washed precipiate containing almost 90-95% of the original radioactivity was suspended in distilled water to give a final volume of about 6.0 ml.

Aliquots of the labeled lithocholyl polylysine were hydrolyzed with equal volumes of 6 N HCl under partial vacuum in special hydrolysis tubes at 105 C for 18 hr. At the end of this period, about 1.0 mg of synthetic unlabeled ϵ-lithocholyl lysine was added to the hydrolysate, which was then diluted with an equal volume of distilled water and extracted twice with the same volume of ethyl acetate to remove traces of free lithocholic acid. The aqueous layer was concentrated to dryness in a rotary evaporator and the residue dissolved in a small volume of a mixture of ethanol-methanol (1:1) for subsequent TLC. Final purification of ϵ-[24-^{14}C] lithocholyl lysine was accomplished by TLC on Silica Gel G, using a mixture of ethanol-ammonium hydroxide-water (6:1:2) as the solvent system.

Preparation of [24-^{14}C] Lithocholyl Bovine Serum Albumin

Lithocholic acid (2.0 mg in 0.5 ml ethanol) and 0.5-2.0 μCi of [24-^{14}C] lithocholic acid were mixed in a 25 ml Erlenmeyer flask and neutralized with 3.5 ml of sodium carbonate (53 mg/100 ml) solution. A solution of bovine serum albumin (7.0 mg in 3.0 ml H_2O) was added dropwise to the mixture while it was stirred vigorously. The coupling was initiated by the addition of a solution of carbodiimide

(8.0 mg in 2.0 ml H_2O). The reaction was allowed to proceed for 24 hr, and then the mixture was dialyzed against several liters of distilled water. The dialyzed preparation was adjusted to pH 9.0 and chromatographed on the anion exchange resin, XAD-2 to remove traces of unconjugated lithocholic acid (14). The effluent from the column, essentially devoid of unconjugated lithocholic acid, was dialyzed and stored at 0-4 C.

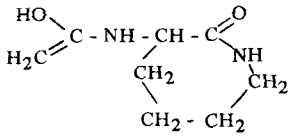
Mass Spectra

Mass spectral data were obtained from a Hitachi-Perkin Elmer RMU 6H mass spectrometer using an ionization voltage of 70 eV.

N-α-Lithocholyl-L-lysine

N-ε-carbobenzyloxy-L-lysine benzyl ester, prepared from N-ε-carbobenzyloxy-L-lysine (15) was coupled with lithocholic acid by means of the mixed carboxylic-carbonic anhydride procedure, using isobutylchloroformate as condensing agent (16). The resulting amide was subjected to hydrogenolysis with 10% palladium on charcoal. After removal of the catalyst by filtration over celite, the filtrate was concentrated to a small volume, and the product was isolated as a white solid by precipitation with ether. Further trituration with ether yielded a white crystalline powder, melting at 225 C. $[\alpha]_D^{26°}$ + 20.83 (C 0.5 methanol), IR (KBr) 3340 cm^{-1} (OH,NH stretch), 2930 cm^{-1}, 2860 cm^{-1} (NH stretch, CH_2), 1710 cm^{-1} (C=O stretch, amide or carboxylic acid), 1620 cm^{-1} (NH deformation), 1580 cm^{-1} (NH deformation, amine), 1450 cm^{-1} (CH_2), 1380 cm^{-1} (CH_2), 1035 cm^{-1} (C-O stretch, hydroxyl).

Mass spectral analysis showed the presence of the molecular ion at *m/e* 504 and a peak at *m/e* 486 due to the formation of the lactam (17). The base peak at *m/e* 170 corresponding to the following structure (McLafferty rearrangement)



is derived from the steroid lactam at *m/e* 486 (17). The most abundant fragment at *m/e* 183 is the result of a cleavage at the C_{21} carbon of the steroid moiety. There is a peak at *m/e* 127 corresponding to a fragment formed by cleavage of the steroid amide lactam (*m/e* 486) at the carboxamide link. An *m/e* 471 fragment resulting from the loss of a methyl group from the steroid lactam at *m/e* 486 is also noticeable.

TLC on Silica Gel G using five different solvent systems gave single steroid positive zones (manganese chloride, sulfuric acid spray). These zones were negative to ninhydrin. Enzymatic hydrolysis with cholyglycine hydrolase yielded lithocholic acid, confirmed by TLC, GLC, and infrared spectroscopy.

N-ε-Lithocholyl-L-Lysine

N-α-carbobenzyloxy-L-lysine benzyl ester (15) was coupled with lithocholic acid through the mixed carboxylic-carbonic anhydride procedure (16) as described by Agarwal and Dhar (18). The resulting amide was subjected to hydrogenolysis with 10% palladium on charcoal catalyst. After removal of the catalyst by filtration over celite, the methanolic filtrate was concentrated to a small volume, and the product, a white crystalline solid, was obtained by addition of ether. MP 162 C $[\alpha]_D^{26°}$ + 18.5[(C 0.5, methanol), IR (KBr) 3340 cm^{-1} (OH), 2920 cm^{-1}, 2860 cm^{-1} (NH stretch, CH_2), 1620 cm^{-1} (NH deformation), 1570 cm^{-1} (NH deformation and CH stretch), 1445 cm^{-1}, and 1375 cm^{-1} (CH_2).

Mass spectral analysis showed the presence of the molecular ion at *m/e* 504 and a peak at *m/e* 487 due to loss of OH. The base peak occurred at *m/e* 144 corresponding to free lysine minue two protons. Other fragments were observed at *m/e* 459 (M-COOH), 445, 442, 427, 358, 215, 213, 199, 186, 171, 157, 145. As expected, there was no evidence for the formation of a lactam.

TLC using five different solvent systems gave single steroid positive spots (manganese chloride, sulfuric acid spray). Unlike the α-substituted conjugate, this conjugate gave a ninhydrin positive spot. Enzymatic hydrolysis with cholylglycine hydrolase gave lithocholic acid.

N-α-ε-Bis-Lithocholyl-L-Lysine

L-lysine benzyl ester ditosylate, prepared according to the procedure of Isumiya and Nakasumi (19), was coupled with two equivalents of lithocholic acid using isobutyl chloroformate as condensing agent in the mixed anhydride procedure in the presence of four moles of triethylamine (16). Hydrogenolysis of the benzyl ester with 10% palladium on charcoal, over night, ensured complete removal of the blocking group. The catalyst was removed by filtration over Celite, and the

filtrate was concentrated almost to dryness. The residue was triturated with ether when a fine white crystalline product separated. This was redissolved in methanol and crystalized with ether to give the product which melted at 152 C. $[\alpha]_D^{26°}$ + 30.18 (C 0.5, methanol). IR was similar to both α and ε-substituted lithochoyl lysines.

TLC showed a single spot, positive for the steroid moiety and negative with ninhydrin. Enzymatic hydrolysis with cholylglycine hydrolase yielded lithocholic acid, α-lithochoyl lysine, and ε-lithocholyl lysine.

RESULTS AND DISCUSSION

Analysis of Human Liver for Soluble Lithocholate (SL) and Tissue-Bound Lithocholate (TBL)

Livers obtained at random from hospital autopsies without regard to diagnoses were subjected to the differential analysis of soluble and tissue-bound lithocholic acid by the procedure described under Methods. Table I presents data for twelve samples of human liver. With the exception of three samples, all of them had varying amounts of soluble (extractable) lithocholic acid, the values ranging from 10-175 μg/g wet weight of liver. Tissue-bound lithocholic acid was uniformly present in all samples and the values ranged from 4-116 μg/g of liver weight. In six of the tissue samples, this represented more than 50% of the total tissue bile acids. In one sample (72-M-159), the concentration of tissue-bound lithocholic acid was as high as 166 μg/g while the extractable fraction had only about 20 μg/g of the bile acid. Since bound lithocholic acid is proportionately greater than the soluble fraction in several liver samples, it is unlikely that bound lithocholic acid is a carry-over from an incomplete extration procedure. Furthermore, control experiments revealed that cholylglycine hydrolase is essential for the release of bound lithocholate, indicating the involvement of a peptide link between the bile acids and protein.

TABLE I

Distribution of Soluble and Bound Forms of Lithocholic Acid in Human Liver[a]

	Lithocholic acid μg/g tissue		Bound lithocholic acid
Tissue number	Soluble	Bound	% of total tissue lithocholic acid
72-M-193	17.6	29.7	62.8
72-M-161	22.6	7.66	25.3
72-M-158	Trace	29.3	100
72-M-159	20.5	116	84.9
72-M-163	43.7	9.1	17.2
72-M-220	39.8	48.9	55.1
A-24-74 S	77.8	4.8	5.8
A-18-74 S	175	10.6	5.72
72-M-144	86.1	24.6	22.2
72-A-52	Trace	11.2	100
72-A-46	10.4	4.4	29.7
72-A-157	Trace	10.9	100

[a]Approximately 50-100 mg of liver tissue from autopsy specimens were homogenized and extracted according to the procedure described under Methods. No pathological data were available for these specimens.

Analysis for Lithocholic Acid Sulfates in Liver

Since lithocholic acid is known to occur in human liver in the form of sulfates, several liver samples were analyzed for this bile acid, with and without solvolysis (20). The livers were homogenized, separated into soluble and bound fractions, and hydrolyzed with cholylglycine hydrolase. Bile acids recovered from this step were divided into two equal aliquots. One was solvolyzed according to the procedure of Palmer and Bolt (20), subjected to TLC, and then processed for GLC to yield total lithocholic acid. The other fraction was treated in an identical manner without the solvolysis step. The procedure involving solvolysis gave values for lithocholic acid both in the sulfated and nonsulfated forms, while the one without solvolysis gave that which was in the nonsulfated form.

The results of solvolysis showed that almost all of the tissue bound lithocholate is present in the nonsulfated form. In the soluble fraction, there is a considerable variation in the distribution of lithocholic acid between its sulfated and nonsulfated forms, although there is an apparent preponderance of the sulfated form as would be expected. Detailed studies on the significance of sulfated lithocholate in the soluble and tissue-bound forms are in progress.

Hydrolysis of [24-^{14}C] Lithocholic Acid from [24-^{14}C] Lithocholyl-Bovine Serum Albumin in the Presence of Liver Tissue

Since cholyglycine hydrolase (peptide bond hydrolase from *C. Perfringens* - ATCC 19574, specific for bile acid conjugates) releases tissue-bound lithocholic acid, the evidence indicated the existence of a peptide link between the bile acid and presumably the side chain NH_2 groups in tissue proteins. Bovine serum albumin (BSA) coupled with [24-^{14}C] lithocholic acid was used as a model to verify the ability of cholylglycine hydrolase to cleave

TABLE II

Enzymatic Cleavage of [24-^{14}C] Lithocholic Acid from [24-^{14}C] Lithocholyl Polylsine Using Clostridial Cholylglycine Hydrolase[a]

Experimental conditions	[24-^{14}C] lithocholic acid isolated (dpm)	
	Ether extract of pH 9.0 mixture	Ethyl acetate extract of pH 1.0 mixture
Control	42	83
Hydrolase	96	2380

[a]An aliquot of [24-^{14}C] lithocholyl polylysine (3000 dpm) was incubated at 37 C for 30 min with and without (control) cholylglycine hydrolase in the presence of acetate buffer, pH 5.8, and co-factors (11). After completing the incubation, nonradioactive lithocholic acid (0.5 mg in 0.1 ml of ethanol) was added to each container, and the mixture was diluted with distilled water to a volume of 25 ml. The pH of the solution was adjusted to 9.5 with 1 N NaOH, extracted twice with equal volumes of ether, and the aqueous layer reacidified to pH 1.0 with 6.0 N HCl. The acidified solution was extracted twice with equal volumes of ethyl acetate. Both organic solvents were evaporated and the radioactivity in the residue determined by liquid scintillation counting. This experiment, repeated several times with different batches of cholylglycine hydrolase, gave similar results.

TABLE III

Thin Layer Chromatography of Lysyl Conjugates of Lithocholic Acid

Compound	R_f				
	Solvent systems[a]				
	A	B	C	D	E
Lithocholic acid[b]	0.65	0.63	0.77	0.80	0.74
α-lithocholyl-L-lysine[b,c]	0	0	0.54	0.45	0.039
ε-lithocholyl-L-lysine[b,d]	0	0	0.49	0.73	0.039
Bis-α-ε-lithocholyl-L-lysine[b,c]	0	0	0.75	0.84	0.48

[a]Solvent systems A: Chloroform-t-amyl alcohol (7:3). B: Iso-amyl acetate-propanol saturated with H_2O (2:1). C: n-butanol-acetic acid-water (4:1:1). D: Ethanol-ammonium hydroxide-water (6:1:2). E: Iso-octane-isopropyl ether-isopropyl alcohol-acetic acid (2:1:1:1) (22).

[b]Sprayed with $MnCl_2$, $4H_2SO_4$ according to Goswami and Frey (23). Viewed under ultraviolet light.

[c]Ninhydrin negative.

[d]Ninhydrin positive.

side chain-linked lithocholic acid, under our experimental conditions in the presence of liver homogenate. BSA-[24-^{14}C] lithocholic acid (11, 417 DPM) was added to 100 mg of human liver, homogenized with 10 ml of 95% ethanol-ammonium hydroxide (1000:1), heated in a water bath at 95 C for 10 min, and centrifuged. The supernatant, which contained most of the albumin-linked radioactive lithocholate, was evaporated to dryness in a rotary evaporator. The residue, dissolved in 0.5 ml of ethanol, was diluted with 0.2 ml of water and hydrolyzed with cholylglycine hydrolase as described in the experimental section. Free lithocholic acid isolated from the acidified medium (pH 1.0) had an activity of 8109 DPM, a recovery of about 71%. By extending the incubation time with cholylglycine hydrolase to 120 min, the recovery could be increased to 85%.

Enzymatic Cleavage of [24-^{14}C] Lithocholic Acid from [24-^{14}C] Lithocholyl Polysine, using Cholylglycine Hydrolase

Radioactively labeled lithocholyl polylysine is a model substrate possessing a well-defined structure in which labeled lithocholic acid is coupled uniformly through amide bonds to the ε-amino groups of lysine in the polypeptide chain. [24-^{14}C] lithocholyl polylysine was hydrolyzed with cholylglycine hydrolase under optimum conditions. The hydrolysate was extracted at pH 9.0, then acidified to pH 1.0 for isolating free, labeled lithocholic acid by reextraction with ethyl acetate. The results showed that free [24-^{14}C] lithocholic acid is released only in the presence of cholylglycine hydrolase (Table II). The results of this exper-

iment and that of the previous experiment with BSA-[24-^{14}C] lithocholate establish that cholylglycine hydrolase can liberate free lithocholic acid from its conjugates with protein via the free ε-NH_2 groups of basic side chains. By analogy, it is assumed that lithocholic acid in tissues, conjugated to basic side groups of amino acids such as lysine, could be cleaved by the use of this enzyme.

However, in previous studies using highly purified cholylglycine hydrolase, we observed very little hydrolysis of deoxycholic acid from synthetic poly-ε-deoxycholyl lysine (10). This apparent anomaly is attributable to the fact that unlike the crude enzyme, highly purified cholylglycine hydrolase is devoid of other nonspecific peptidases which would cleave the large polypeptide chains into smaller fragments, making it accessible to the cholanoyl amino acid hydrolase. Furthermore, synthetic substrates such as poly-ε-deoxycholyl lysine are extremely insoluble fully substituted polymers providing limited steric access to hydrolytic cleavage with our enzyme. Similar effects of steric hindrance arising from the n-ε-alkyl groups of lysine have been described by Moore and Benoiton (21). Presumably, we would not expect to find fully substituted (with lithocholic acid) polylysine sequences in liver.

Identification of ε-Lithocholyl Lysine in Human Liver Tissue

Aliquots of human liver, known to contain high concentrations of tissue-bound lithocholic acid, were extracted with ethanol-ammonia by the procedure described earlier. The residue from this step, when hydrolyzed with 6 N HCl under partial vacuum and then chromatographed on Silica Gel G plates with ethanol-ammonium hydroxide-water (6:1:2) as the mobile phase, showed a ninhydrin positive spot (Rf-0.72) with a migration similar to that of authentic ε-lithocholyl lysine (Rf-0.73) (Table III). This zone was also positive for bile acids when sprayed with $MnCl_2/H_2SO_4$ and viewed under ultraviolet light (23). Since among the three synthetic lithocholyl lysines only ε-lithocholyl lysine gives a positive ninhydrin reaction, the unknown compound was tentatively identified as ε-lithocholyl lysine. Moreover, the normal conjugates of lithocholic acid (with glycine and taurine) have different migrations and are ninhydrin negative. The material extracted from the zone corresponding to ε-lithocholyl lysine upon co-chromoagraphy with ε-[24-^{14}C] lithocholyl lysine showed that all of the radioactivity was recoverable from the ninhydrin positive zone. Myher et al. (17) have shown that synthetic N-α-cholylornithine is ninhydrin negative while the natural ornithocholanic acids (4) are ninhydrin positive. It appears that in the natural product, cholanic acid is linked via the side chain of ornithine.

In replicate experiments, the unknown compound isolated from liver was purified by TLC as described above and hydrolyzed with cholylglycine hydrolase. The presence of lithocholic acid in the hydrolyzate was demonstrated by GLC of the methyl ester trifluoroacetates. No lithocholic acid was detected in unhydrolyzed controls. Infrared and mass spectrometry of the purified product showed the presence of ε-lithocholyl lysine. Using tissue extracts for TLC, zones with migrations similar to that of authentic ε-lithocholyl lysine and bis-α-ε-lithocholyl lysine (Table III) upon hydrolysis with cholylglycine hydrolase and subsequent GLC did not show the presence of lithocholic acid. The combined results of these studies showed that ε-lithocholyl lysine is the predominant residue of lithocholate in tissue-bound form.

Stability of ε-Lithocholyl Lysine and Lithocholyl Polylysine to 6 N HCl Hydrolysis

Since we used 6 N HCl for the hydrolysis of tissue proteins, it was desirable to determine the effect of this procedure on the stability of the lithocholyl lysine bond under these conditions. Approximately 2.0 mg of ε-lithocholyl-L-lysine was hydrolyzed under nitrogen with 2.0 ml of 6 N HCl at 110C for about 6 hr. The reaction mixture was evaporated under partial vacuum over 1 N KOH, and the residue, dissolved in a small volume of ethanol, was subjected to TLC using chloroform-t-amyl alcohol (70:30) as the mobile phase. Free lithocholic acid was not present in detectable amounts when the plate was exposed to iodine vapors. GLC of the zones corresponding to free lithocholic acid revealed a 5% hydrolysis of the conjugates under these conditions.

Lithocholyl polylsine, when hydrolyzed under the same conditions, did not show the release of any free lithocholic acid. However, there was a ninhydrin positive zone at the origin corresponding to ε-lithocholyl-L-lysine. This zone would also have any α-lithocholyl-L-lysine released from the N-terminal end.

The demonstration of a lysine residue in either native or synthetic lithocholyl lysine was technically difficult because of the unusual chemical stability of the substance towards acids. Enzymatic cleavage of the bond

would yield a mixture of amino acids from the enzyme preparation rendering the results inconclusive.

On the Nature of Tissue-Bound Lithocholic Acid

Our studies have shown that lithocholic acid occurs in two forms in human liver, one that is readily solubilized by ethanol-ammonia and the other which remains bound to the tissue residue. Although ethanol-ammonia has been used for extracting tissue bile acids, both free and conjugated (8), this solvent system may not be highly selective in differentiating other conjugates of lithocholic acid with polypeptides of low molecular weight. Therefore, the terms "soluble" and "tissue bound" are operational terms relative to their behavior towards ethanol-ammonia, and at this stage cannot be defined in strict chemical terms.

Hydrolysis of tissue residues with 6 N HCl and subsequent TLC revealed the presence of ϵ-lithocholyl lysine. However, when [24-^{14}C] lithocholyl polylysine was similarly hydrolyzed and subjected to TLC, the recovery of ϵ-[24-^{14}C]lithocholyl lysine was not quantitative, since a significant amount of radioactivity remained at the origin during chromatography. It appears, therefore, that 6 N HCl is only partially effective in the hydrolysis of peptides and proteins to which lithocholic acid is linked via side chain amide bonds.

Pathophysiological Significance of Lithocholic Acid

Lithocholic acid and other related monohydroxylated bile acids as a class show unique toxic properties that are not seen with other dihydroxy or trihydroxy bile acids. Monohydroxy bile acids have been shown to affect organs, cells, and subcellular organelles in a variety of ways. Lithocholate induces an inflammatory response and is pyrogenic when injected subcutaneously (24). In the liver, its hepatotoxicity is manifested by cholestasis, bile duct proliferation, and cirrhosis; the exact molecular mechanisms of these effects are not clearly understood (25). Based on the physicochemical properties of the monohydroxylated bile acids, and on their relatively greater lipophilic character, several investigators have suggested a direct effect on the lipid constituents that maintain the functional and structural integrity of cellular membranes (25). Similarly, lithocholates have been shown to bind to proteins with an affinity inversely proportional to the number of hydroxyl substituents on the steroid (26) in spite of the fact that monohydroxy bile acids are normally insoluble in protein-free aqueous systems. It should also be emphasized that the identification of tissue-bound lithocholic acid in human liver poses important questions about the role of lithocholic acid in liver injury and disease. The absence of lithocholic acid sulfate in tissue-bound form is also interesting because of the widely-held view that sulfation is an intrinsic, protective mechanism against liver injury by toxic monohydroxy bile salts. It is premature to ascribe any pathognomonic significance to tissue-bound lithocholic acid at this stage and more data will be needed to establish the true effects of TBL at the cellular level.

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Substrate Metabolism in the Perfused Lung: Response to Changes in Circulating Glucose and Palmitate Levels[1]

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ABSTRACT

The effects of circulating levels of glucose and palmitate in the isolated perfused rat lung were investigated. Rat lungs were perfused for 1.5 hr with washed bovine erythrocytes (15% hematocrit) in Krebs-Henseleit bicarbonate buffer containing 5 g% bovine serum albumin. Glucose uptake in the perfused lung varied directly with circulating glucose concentration. Lactate production was affected proportionately more by high glucose levels than by low concentrations. Pyruvate production was decreased by both low glucose and palmitate concentration in the circulating medium. Oxidation of glucose to CO_2 was depressed by low glucose and by high palmitate concentrations. Glucose incorporation into lung lipids was more strongly influenced by glucose concentration than by circulating palmitate levels. These data indicate acute changes in circulating levels of glucose and palmitate alone can act to either inhibit or stimulate glycolysis, glucose oxidation, and lipid synthesis in the perfused lung.

INTRODUCTION

The rat lung lacks a large substrate or energy reserve (1,2) to maintain essential biosynthetic activity and, therefore, is highly dependent on the circulating blood. Although a variety of nutrients can be utilized by the lung (3), glucose and fatty acids, due to their important role in lipogenesis (4-8) and relatively high uptake and/or utilization, constitute two major substrates used by the lung.

Both glucose and palmitate metabolism in the lung have been shown to be sensitive to altered physiologic states. In one such condition, lungs from fasted rats had decreased lipid enzymatic activity (9), depressed glucose incorporation into lung lipids and oxidation to CO_2 (10,11), while palmitate oxidation to CO_2 and incorporation into lung lipids were accelerated (10). In another type of altered physiologic state, lungs made hypoxic for 24 hr showed increased glucose utilization (12) and depressed palmitate incorporation into lung lipids (13).

In both the fasted and hypoxic condition, circulating substrate concentration is drastically altered. Plasma glucose was decreased and fatty acid levels were elevated in fasting (14), while glucose levels were elevated (12) and serum fatty acids were unchanged in hypoxia (13). Thus, the changes seen in lung glucose and palmitate metabolism during these conditions could be due, in part, to altered circulating substrate levels. At present, very little information is known regarding the interaction between circulating substrate concentrations and lung metabolism. The present investigation was undertaken to examine the way in which circulating glucose and palmitate levels regulate pattern of substrate utilization and lipid synthesis in the perfused lung.

METHODS

Male Long-Evans Hooded rats weighing ca. 300 g were heparinized (1 unit · g body wt^{-1} IP) 15 min prior to anesthetization with sodium pentobarbital (6 mg · 100 g body wt^{-1}, IP). Animals were sacrificed by exsanguination via the carotid artery. The trachea was cannulated in situ and closed immediately prior to thoracotomy trapping air in the lung. The apex of the heart was cut; the inflated lungs and heart were excised en bloc and weighed. The pulmonary artery was cannulated, and the left atrium cut to allow the perfusing medium to return for recirculation. The lung's vasculature was flushed via the arterial cannula with Krebs-Henseleit bicarbonate buffer (KHB) (15). The lung was then suspended by the trachea, placed in a moist organ chamber, and maintained at 37 C in a temperature controlled lucite box containing a circulating fan (see Fig. 1). Further details of the isolated perfused lung system have been described elsewhere (8,16).

The perfusion medium consisted of washed bovine red blood cells resuspended to a 15% hematocrit with KHB containing 5 g% bovine serum albumin (Pentex BSA, Fraction V, Miles Laboratories). The bovine blood was collected in a 20% acid-citrate-dextrose solution, centrifuged (250 x G), serum aspirated, and the packed erythrocytes stored at 205 C and used within 48 hr of collection. On the day of each experiment, the erythrocytes were

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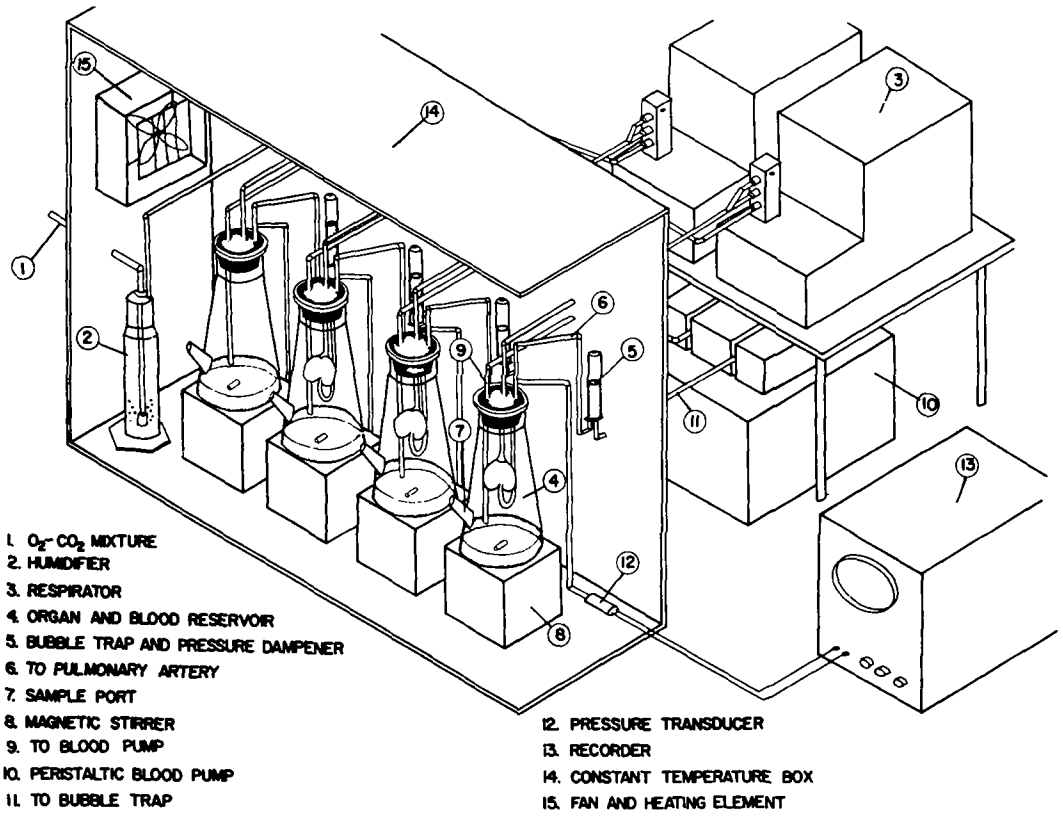

FIG. 1. Schematic of perfused lung preparation with numbers indicating the various components. Ventilation was 100 cycles/min and perfusate flow was 10 ml/min.

washed three times with approximately equal volumes of KHB. Palmitic acid (Sigma Chemical Co.) was bound to the albumin and the pH adjusted to 7.4 with 0.8M sodium carbonate (8). The KHB-albumin-palmitate solution and erythrocytes were combined and the resulting perfusion medium equilibrated with 21% O_2, 74% N_2, and 5% CO_2 while being brought up to 37 C. Seventy ml of the perfusate were placed in each of the specially designed Erlenmeyer flasks fitted with sidearms. The perfusate was mixed gently with a magnetic stirrer and circulated at a flow rate of 10 ml/min. Lungs were ventilated with 21% O_2 75% N_2, and 5% CO_2 at a frequency of 100 cycles/min, a minute volume of 60 ml/min, and a positive end expiratory pressure of 2 cm of water.

One ml of isotopic solution containing 20 μCi of [U-^{14}C] glucose (sp. act. 15 μCi/μmole) and 25 μCi of [9,10-^{3}H] palmitate bound to albumin (sp. act. 330 μCi/μmole) was added as a single pulse to the medium. All-lungs were then perfused for 5 min to allow for equilibration before initial samples were taken. A 10 ml sample was withdrawn from the blood reservoir at the beginning and end of the 1.5 hr perfusion period.

Net uptake and production of metabolites were calculated as a product of initial and final concentration difference and perfusate volume. The red blood cell contribution was accounted for at each substrate concentration by circulating a known volume through a set up without the lung and measuring initial and final concentrations. Subtraction of the contribution of the erythrocytes from the lung plus erythrocytes yielded the net contribution by the isolated perfused lung. Initial and final samples of perfusate were assayed for glucose (17), lactic acid (18), and pyruvate (19). Perfusate $^{P}O_2$, $^{P}CO_2$, and pH were measured with a Corning blood gas analyzer. Perfusate fatty acids were measured by the method of Itaya and Ui (20). An additional 5 ml of perfusate was collected at the end of the perfusion period for liberation and trapping of $^{14}CO_2$. During the perfusion period, a trapping apparatus for collecting CO_2 was attached to the exhaust port of the respiration pump (16). All $^{14}CO_2$ samples were resuspended in a scintillation fluid prepared according to Buhler (21). At the end of the perfusion period, the pulmonary vasulature was

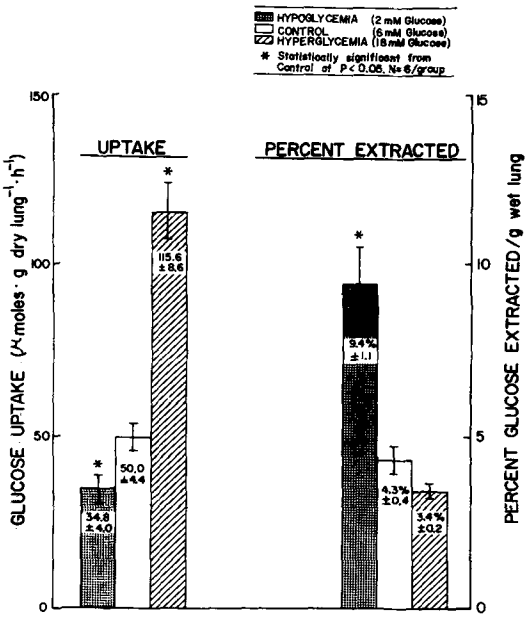

FIG. 2. Influence of circulating glucose concentration on glucose uptake by perfused lungs. Lungs were perfused for 1.5 hr at a flow rate of 10 ml/min and were ventilated with 21% O_2 and 5% CO_2. Values are mean ± SE with N=6/group. Percent glucose extracted was calculated as (initial - final difference x g wet wt^{-1} x initial $concentration^{-1}$) x 100.

flushed with physiological saline to remove the radioactive perfusate. Post-perfused lungs were analyzed for percent water and lipid radioactivity. Lung lipids were extracted, separated into lipid classes, and phospholipids subjected to mild alkaline hydrolysis as previously described (22). Scintillation fluid for total lipids, neutral lipids and free fatty acids, phospholipids, and phospholipid-fatty acids was a toluene-based solution with 4.0 g Omnifluor (Pilot Chemicals, Inc.) Inc.) per liter solution Omnifluor: Triton X-100 [(Rohm and Haas) 2:1, v/v] scintillation fluid was used for resuspension of the phospholipid-glyceride-glycerol moiety from hydrolysis. All radioactive samples were subjected to radioassay using a Tri-carb 3375 scintaillation spectrometer (Packard Instruments).

After comparison of variances using an F-test, Student's t-tests (pooled or unpooled) were utilized for analysis of differences between experimental and control means. The 95% level of confidence was accepted as the criterion for statistical significance.

RESULTS

Glucose uptake (initial-final concentration difference) and the percent extracted from the medium [(initial-final difference x wet wt^{-1} x initial $concentration^{-1}$) x 100] were strongly influenced by high and low glucose levels (Fig. 2). Glucose uptake varied directly with glucose concentration while percent extracted varied inversely with glucose concentration. The percent glucose extracted showed more than a twofold increase in the "hypoglycemic lungs." Although not shown, palmitate levels did not affect glucose uptake or percent extracted. Circulating levels of glucose and palmitate also had a significant influence on lactate and pyruvate production in the perfused lung (Table I). Lactate production was significantly affected by 18 mM glucose (50% increase), while pyruvate production was reduced by both hypoglycemia (20% decrease) and hypolipemia (38% decrease). The lactate-pyruvate ratio (L/P), an indication of the redox state of the cell, increased 37% with the "hyperglycemic" condition and 98% with the "hypolipemic" group. Oxidation of glucose to CO_2 by the perfused lungs was markedly depressed (30%) in both the "hypoglycemic" condition and the "hyperlipemic" condition (Table II), and indicates that carbon flow through the citric acid cycle is influenced by both circulating glucose and palmitate levels.

As seen in Table II, [^{14}C] glucose incorporation in total lung lipids (TL) was strongly influenced by altered glucose concentration, while palmitate concentration did not affect glucose incorporation into lung lipids. Utilization of [^{3}H] palmitate in TL, however, was significantly altered with palmitate concentration and also by low glucose levels. The effect of exogenous substrate levels on utilization of glucose and palmitate in neutral lipids (NL) and phospholipids (PL) is presented in Table III. Circulating glucose levels had a greater effect on [^{14}C] glucose incorporation into NL than PL. Similarly, circulating levels of palmitate also affected [^{3}H] palmitate and [^{14}C] glucose incorporation more in NL than in PL, and suggest that PL synthesis from glucose and palmitate at high circulating levels is limited more by carbon flow through the PL synthetic pathway than by substrate concentration. Incorporation of both ^{14}C-glucose and ^{3}H-palmitate into NL increased with increasing palmitate levels. Utilization of these substrates in PL was affected only with the lowest palmitate concentration. It should be noted that with hypolipemia, palmitate incorporation in PL decreased 61%, while glucose incorporation in PL increased 33%. Further separation of PL into fatty acid (PLFA) and glyceride-glycerol (PLGG) moieties (Table IV) show that incorporation of C^{14}-glucose into PLFA varied directly with

TABLE I

Effect of Exogenous Substrate Levels on Lactate and Pyruvate Production in Perfused Lung[a]

Substrate concentration	(μmoles · dry lung^{-1} · hr^{-1}) Lactate production	Pyruvate production	L/P
Glucose			
2 mM	68.1 ± 3.6	8.0*± 0.5[b]	8.6 ± 0.4
6 mM	86.3 ± 7.9	10.0 ± 0.6	8.6 ± 0.4
18 mM	131.5*± 9.4	11.1 ± 1.6	11.8*± 0.7
Palmitate			
0.2 mM	99.0 ± 11.2	6.2*± 0.8	17.0*± 2.0
0.7 mM	86.3 ± 7.9	10.0 ± 0.6	8.6 ± 0.4
1.8 mM	91.1 ± 10.0	11.1 ± 0.8	8.1 ± 0.5

[a]Values are mean ± SE. Fatty acid concentration in circulating medium was 0.7 mM when glucose concentrations were manipulated. Glucose concentration in circulating medium was 6 mM when fatty acid concentrations were manipulated. N = 6/substrate concentration.

[b]*Statistically significant from control (6 mm glucose; 0.7 mM palmitate) at $P < 0.05$.

TABLE II

Effect of Circulating Substrate Levels on Utilization of [^{14}C] Glucose and [^{3}H] Palmitate[a]

Substrate concentration	[^{14}C] Glucose converted to CO_2	[^{14}C] Glucose converted to Total lipids	[^{3}H] Palmitate converted to Total lipids
Glucose			
2 mM	3.70 ± 0.45*[b]	1.59 ± 0.12*	8.86 ± 0.36*
6 mM	5.28 ± 0.33	2.88 ± 0.16	9.97 ± 0.29
18 mM	5.73 ± 0.48	3.68 ± 0.23*	11.18 ± 0.55
Palmitate			
0.2 mM	5.01 ± 0.30	3.03 ± 0.16	3.37 ± 0.21*
0.7 mM	5.28 ± 0.33	2.88 ± 0.16	9.97 ± 0.29
1.8 mM	3.68 ± 0.49*	3.10 ± 0.09	14.50 ± 0.42*

[a]Values are mean ± SE and are expressed μmoles substrate converted · g dry lung^{-1} 1 H^{-1}. N = 6/substrate concentration.

[b]*Statistically significant from control (/ mM glucose; 0.7 mM palmitate) at $P < 0.05$.

increasing glucose concentration. However, glucose incorporation into PLFA is inversely related to circulating palmitate concentration. Circulating palmitate levels appeared to exert a stronger influence on glucose incorporation into PLFA than did circulating glucose levels (Table IV). Utilization of [^{3}H] palmitate in PLFA increased significantly only with the highest glucose levels and was markedly depressed (61%) only with the lowest palmitate levels. Glucose incorporation into PLGG was significantly decreased from control during 2 mM glucose perfusions.

DISCUSSION

The present study demonstrates that changes in circulating substrate levels alone play an important role in regulating lung metabolism. In particular, glucose uptake by the lung appears to be concentration dependent. The correlation between concentration and uptake seen in Figure 2 is especially evident and suggests that circulating concentration may be a very important factor in regulating glucose transport and phosphorylation in the lung since this organ appears to be insensitive to insulin (5,6).

Lung lactate production is also related to glucose concentration. While hypoglycemia did not significantly affect lactate production (Table I), hyperglycemia caused a 52% increase in lactate production. Since increased circulating glucose levels can act to increase enzymatic activity with a concomitant increase in glycolysis (23), such a mechanism would explain the increased lactate production with hyperglycemia. Moreover, elevated glucose concentration may also explain, in part, the proportionately higher lactate production seen in lungs exposed to hypoxia for 24 hr vs. 2 hr, since serum glucose levels in the 24 hr hypoxic animals were significantly elevated (12). Alterations in palmitate levels did not significantly effect lactate production. This observation is substantiated by O'Neil and

TABLE III

Influence of Exogenous Substrate Levels on Perfused Lung Lipid Metabolism[a]

	(μmoles converted · g dry lung^{-1} · hr^{-1}			
	Neutral lipids and free fatty acids		Phospholipids	
Substrate concentration	([^{14}C] Glucose)	([^{3}H] Palmitate)	([^{14}C] Glucose)	([^{3}H] Palmitate)
Glucose				
2 mM	0.29 ± 0.02*[b]	2.86 ± 0.13	1.04 ± 0.07*	4.94 ± 0.24
6 mM	0.62 ± 0.08	3.31 ± 0.28	1.64 ± 0.11	5.08 ± 0.24
18 mM	0.87 ± 0.06*	3.91 ± 0.22	2.03 ± 0.17	5.65 ± 0.31
Palmitate				
0.2 mM	0.39 ± 0.03*	0.83 ± 0.05*	2.19 ± 0.12*	2.24 ± 0.18*
0.7 mM	0.62 ± 0.08	3.31 ± 0.28	1.64 ± 0.11	5.08 ± 0.24
1.8 mM	0.83 ± 0.02*	7.62 ± 0.38*	1.84 ± 0.05	5.56 ± 0.12

[a]Values are mean ± SE. N = 6/substrate concentration. See Table I for details.

[b]*Statistically significant from control (6 mM glucose; 0.7 mM palmitate) at $P < 0.05$.

TABLE IV

Effect of Circulating Substrate Level on Incorporation of [^{14}C] Glucose and [^{3}H] Palmitate into Lung Phospholipid[a]

	(μmoles converted · g dry lung^{-1} · hr^{-1})		
	Phospholipid fatty acid		Phospholipid glyceride glycerol
Substrate concentration	[^{14}C] Glucose	[^{3}H] Palmitate	[^{14}C] Glucose
Glucose			
2 mM	0.11 ± 0.01*[b]	4.51 ± 0.23	1.02 ± 0.07*
6 mM	0.26 ± 0.03	4.89 ± 0.18	1.79 ± 0.14
18 mM	0.40 ± 0.03*	5.49 ± 0.18*	2.13 ± 0.13
Palmitate			
0.2 mM	0.59 ± 0.06*	1.91 ± 0.15*	1.78 ± 0.11
0.7 mM	0.26 ± 0.03	4.89 ± 0.18	1.79 ± 0.14
1.8 mM	0.08 ± 0.01*	5.11 ± 0.08	1.83 ± 0.04

[a]Values are mean ± SE. N = 6/substrate concentration.

[b]*Statistically significant from control (6 mM glucose; 0.7 mM palmitate) at $P < 0.05$.

Tierney (24) who found that the presence of palmitate in the media did not appear to affect lung lactate levels. Thus, the lung appears to be capable of maintaining a steady state of lactate production even when palmitate levels are altered or when glucose levels are in low supply, but can, depending on the stimuli, produce greater quantities of lactate when glucose becomes more readily available. The functional importance of lactate production in an organ that has a high partial pressure of oxygen is not known at this time.

Pyruvate release into the perfusion media was significantly reduced with both hypoglycemia and hypolipemia, and accounts, for the most part, for the increase in L/P ratio. Increases in L/P ratios as seen with hyperglycemia and hypolipemia reflect concomitant elevations of cytoplasmic NADH/NAD ratios and have been associated with inhibition of the citric acid cycle (25). However, this does not appear to be the case in these instances since CO_2 production from glucose in hyperglycemia and hypolipemia did not differ significantly from control. The decreased glucose oxidation to CO_2 seen with high palmitate (Table II) is important in that it demonstrates that elevated serum fatty acids can inhibit glucose oxidation in the perfused lung. In previous studies (10), we have shown that the lung has the ability to switch to palmitate as a preferred oxidative precursor in conditions where serum fatty acids are elevated.

Lipogenesis in the lung is primarily directed toward formation of phospholipids (26,27) in contrast to other organs such as liver and adipose tissue where metabolic pathways are oriented primarily toward the formation of neutral lipids (7,28). Free fatty acids are not only rapidly esterified in the lung, but lung tissue can also oxidize fatty acids (3,5,6,29).

The increase seen in palmitate incorpora-

tion in total lipids and specifically neutral lipids plus free fatty acid (NL) fraction may be a function of fatty acid transport in the cell. However, elevated palmitate levels stimulated glucose incorporation plus palmitate incorporation into this NL fraction. Therefore, while an increase in palmitate incorporation in the NL fraction may have been an indication of increased fatty acid transport, a general stimulation of neutral lipid synthesis was also apparent. Although phospholipid metabolism was less affected than neutral lipids by altered substrate levels, there were, however, striking changes seen in PLFA. For example in hypoglycemia, PLFA was depressed proportionately more than PLGG, and indicate that glucose is being conserved for the formation of α-glycerol phosphate which serves as the acceptor molecule in esterification. It is interesting to note that the lowest percentage of glucose in the glycerol moiety (75%) was found when the circulating palmitate incorporation was lowest (0.2 mM palmitate) and glucose carbons would be needed for fatty acid synthesis. However, as more palmitate was available for phospholipid-fatty acids, proportionately more (75% to 96%) of the glucose carbons were incorporated into the glycerol vs. the fatty acid moiety. Conversely, the percent of glucose in phospholipid-glycerol increased from 84% to 90% with decreasing media glucose levels as relatively more glucose carbons were conserved for the synthesis of the α-glycerol phosphate backbone of essential lung phospholipids. From the foregoing observations, the synthesis of phospholipid-fatty acids from glucose and palmitate appear to be intimately related and is further exemplified during hypolipemia (Table IV) where the largest percent change in palmitate incorporation into phospholipid-fatty acids (a 61% decrease) is accompanied by the largest percent change in glucose incorporation into phospholipd-fatty acids (a 128% increase). Glucose incorporation into phospholipid-fatty acids appeared to be highly variable and more strongly influenced by palmitate levels than by glucose levels. Palmitate has been shown to inhibit acetyl-carboxylase in the lung (9), which suggests decreases in glucose carbons incorporated into PLFA are controlled at this point.

As a concluding remark, these data indicate that acute changes in circulating levels of glucose and palmitate may provide a mechanism for immediate regulation of lung metabolism by changing intracellular metabolite concentrations which either act to inhibit or activate glycolysis, oxidation, and lipid synthesis.

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Hypercholesterolemia and Triglyceride Secretion Rates in Monkeys Fed Different Dietary Fats

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ABSTRACT

The influence of hypercholesterolemia on the triglyceride secretion rate was studied in both squirrel and cebus monkeys fed coconut oil, corn oil, or safflower oil. The triglyceride secretion rate (TGSR) was determined in vivo following the administration of Triton WR1339, which blocks the clearance of very low density lipoprotein (VLDL). Thus, the increase observed in circulating triglyceride after Triton administration presumably reflects hepatic triglyceride (VLDL) secretion in the fasted state. The VLDL-TGSR was lowest in hypercholesterolemic monkeys and highest in those fed unsaturated fat diets and having a low serum cholesterol. In all instances, TGSR was inversely correlated with the plasma cholesterol concentration. While a definitive explanation for these observations must await further investigation, the possibility that circulating low density lipoprotein (LDL) acts to feed back on VLDL secretion is discussed. The decreased TGSR associated with the diet-induced cholesterolemia also implies clearance of VLDL is impaired under these conditions.

INTRODUCTION

It is widely accepted that feeding saturated fat with or without cholesterol to humans and various experimental animals results in elevated concentrations of plasma cholesterol. Generally the hypercholesterolemia is positively correlated with circulating low density lipoprotein (LDL) concentration. Thus, the formation and turnover of this lipoprotein are important considerations for study of the mechanism(s) of the hyperlipemia.

The origin of plasma LDL has been the subject of several investigations, and most of the data from studies in humans (1-3) and non-human primates (4) indicates that greater than 85% of the β apoprotein associated with LDL derives from the catabolism of very low density lipoprotein (VLDL). In fact, the generally observed low concentration of VLDL in non-human primates is thought to result from its rapid turnover to intermediate density lipoprotein (IDL) and LDL (4,5). This precursor-product relationship between VLDL and LDL raised the question whether enhanced synthesis and secretion of VLDL might partially explain the elevated LDL associated with saturated fat feeding.

Studies in hypercholesterolemic gerbils fed saturated fat (6) and squirrel monkeys fed cholesterol (7) have demonstrated reduced hepatic production of VLDL and raised the possibility that increasing levels of LDL cholesterol may inhibit the secretion of its precursor—VLDL. To pursue this possibility and indirectly test the hypothesis that hypercholesterolemia associated with saturated fat feeding may reflect altered clearance of cholesterol-rich lipoproteins, the in vivo rates of VLDL triglyceride secretion (TGSR) of two species of monkeys fed saturated fat (coconut oil) were compared to monkeys fed unsaturated fat (corn or safflower oil) following the injection of Triton WR1339. Since this detergent coats lipoproteins, particularly VLDL, to prevent their catabolism by lipoprotein lipase (8), the increase observed in circulating triglycerides after Triton administration presumably reflects hepatic (VLDL) triglyceride secretion in the fasted state.

MATERIALS AND METHODS

Nine squirrel monkeys (*Saimiri sciureus*) and 16 cebus monkeys (*Cebus albifrons*) of both sexes were divided into three groups and fed our semipurified diets previously described (9) containing either coconut, corn, or safflower oil as the dietary fat. [The semipurified diet was an agar-based cake which contained (in %): lactalbumin, 16: dextrin, 25.5; oil, 8; Hegsted IV salt mix, 4.0; choline chloride, 0.24; inositol, 0.1; vitamin, 0.4. For details of diet preparation, see Ref.9.] Both species of monkeys were fed these diets for 3 years from birth at which time stable body weight and plasma cholesterol concentrations indicated steady state conditions were approximated.

The TGSRs were determined in monkeys deprived of food overnight. The following morning each was injected via the saphenous vein with a solution of Triton WR1339 (p-isooctyl polyoxyethylene phenol polymer; Ruger

TABLE I

Effect of Dietary Fat on Plasma Cholesterol Concentration and Triglyceride Secretion Rates Following Triton Administration

Diet and species	Plasma cholesterol (mg/dl)	VLDL triglyceride secretion rate (mg/hr/kg)
Squirrel		
Coconut oil (3)[a]	311.3 ± 34.0[b]	5.4 ± 0.4
Corn oil (3)	168.0 ± 24.0	7.4 ± 1.4
Safflower oil (3)	87.3 ± 7.0	13.4 ± 0.6
Cebus		
Coconut oil (8)	273.0 ± 16.5	5.2 ± 0.3
Corn oil (3)	141.0 ± 5.2	8.6 ± 1.6
Safflower oil (5)	138.0 ± 16.0	8.6 ± 1.3

[a]Figures in parentheses are the number of animals per group.
[b]Mean ± SEM.

Chemical Co., New York, NY) in 0.9% NaCl (100 mg/ml) at a dose of 250 mg/kg body weight. In preliminary experiments, this dosage resulted in a linear increment of plasma triglycerides for at least 8 hr. Blood samples were withdrawn from the femoral vein at 0, 2, 4, and 6 hr post-Triton into syringes containing 15% EDTA, and the plasma was separated by low speed centrifugation at 4 C. Triglyceride (Dow Diagnostics Reagent Sets: Triglycerides, Indianapolis, IN) and cholesterol concentrations were determined colorimetrically (10). Triton concentration measured colorimetrically (11) after extracting the plasma with isopropanol ranged between 6-7 mg/ml for all diets and remained constant for the duration of the TGSR determinations. The TGSR for each monkey was calculated as previously described from the slope of the least squares regression of plasma triglyceride concentration over time (6). This was multiplied by the estimated plasma volume (assumed to be 45 ml/kg) and divided by the body weight to give the secretion rate in mg/kg body weight/hour.

Analyses of variance and covariance were carried out using the Statistical Package for the Social Sciences (12) while the nonlinear regression was fitted using program BMDP3R, as revised in August 1976, from the BMDP package developed at the UCLA Health Sciences Computing Facility (13). The slopes for TGSR were determined by calculator.

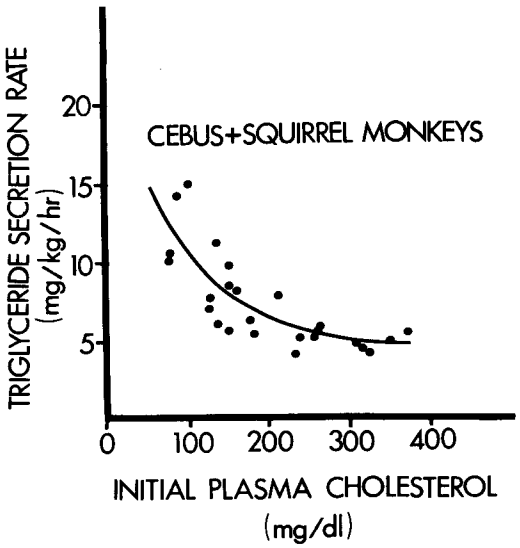

FIG. 1. Individual triglyceride secretion rates (TGSRs) plotted against initial plasma cholesterol concentrations indicate that an inverse, nonlinear relationship exists between these parameters over the range of cholesterol concentrations observed.

RESULTS

Table I shows the fasting plasma cholesterol levels and the VLDL-TGSR for the three dietary groups for both squirrel and cebus monkeys. Analysis of variance of the fasting cholesterol values revealed a clear diet effect, but no significant species effect nor a species x diet interaction. Duncan's multiple range test (14) indicated that a significant diet difference existed between coconut oil and the other two dietary fats, but the latter did not differ significantly from each other.

Inspection of Table I revealed that the lowest TGSR was associated with the highest levels of plasma cholesterol (the coconut oil diets in both species) while the highest TGSR was associated with the lowest plasma cholesterol values (safflower oil diet fed to squirrel monkey). Analysis of variance for TGSR indicated a highly significant diet effect, and a significant species x diet interaction. This interaction was due to the marked difference in the response of the two species to the safflower oil

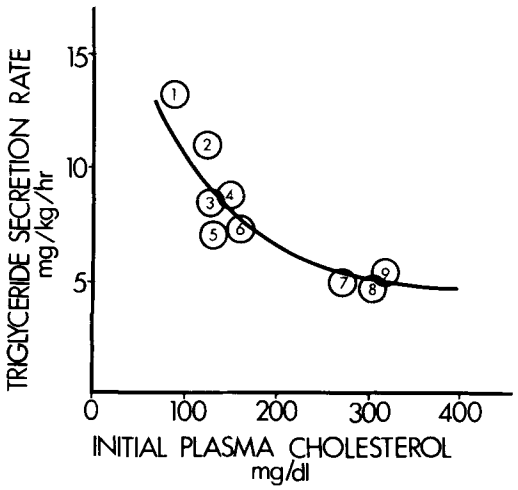

FIG. 2. Mean values of triglyceride secretion rate (TGSR) vs. plasma cholesterol concentration obtained from the literature and fitted to the curve generated by data in Figure 1. Values represent a variety of dietary circumstances in nonhuman primates as follows: 1 = squirrel monkeys fed safflower oil; 2 = squirrel monkeys fed corn oil (7); 3 = cebus monkeys fed corn oil; 4 = cebus monkey fed safflower oil; 5 = rhesus monkeys fed commercial monkey chow (15,16); 6 = squirrel monkeys fed corn oil; 7 = cebus monkey fed coconut oil; 8 = squirrel monkey fed coconut oil; 9 = squirrel monkey fed corn oil + cholesterol (7).

diet, in contrast to their similar responses to the other two diets. Analysis of covariance to determine how much of the observed differences in TGSRs were attributable to plasma cholesterol concentrations revealed that this variable, i.e., plasma cholesterol, accounted for all observed differences in TGSRs due to species or diet.

A plot of the relationship between plasma cholesterol and TGSR for all 25 animals (Fig. 1) showed an inverse association existed between these two variables. The TGSR declined with increasing cholesterol levels to the approximate range of 220-250 mg/dl after which it tended to stabilize. The relationship was expressed by a single exponential equation which covers both the initial decline and the apparent stabilization of the curve:

$$TGSR = 4.44 + 17.8\, e^{-0.0106\, chol}$$

where e is the base of the natural logarithms (= 2.71828. . .).

Figure 2 shows the closeness of fit to the above curve of mean TGSR values from our six groups of New World monkeys and from published results of other investigators studying both New World (7) and Old World (15,16) monkeys.

DISCUSSION

The data reported here, in agreement with previous findings in hypercholesterolemic gerbils and nonhuman primates fed saturated fat or dietary cholesterol, respectively (6,7), demonstrate that squirrel and cebus monkeys made hypercholesterolemic by coconut oil feeding had reduced rates of triglyceride secretion into plasma following the administration of Triton WR1399 when compared to normocholesterolemic monkeys fed either corn or safflower oil. The secretion rates of triglyceride reported in this communication were interpreted to represent rates of secretion of VLDL since recent investigations (7) revealed that the newly secreted plasma triglyceride in Tritonized squirrel monkeys was found exclusively contained in the VLDL fraction. Furthermore, as other studies have indicated that the liver is the primary source of plasma triglyceride in the postabsorptive state (17-19), the VLDL secreted in our fasted monkeys were assumed to be predominantly of hepatic origin.

Results plotted in Figure 1 indicate an inverse correlation between plasma cholesterol levels and TGSRs. Although this association does not necessarily imply a product-precursor feedback inhibition by LDL on VLDL secretion, it is at least suggestive since those animals having similar plasma cholesterol values, independent of dietary fat and species, also had similar TGSRs whereas monkeys having dissimilar plasma cholesterol levels, yet fed the same fat, had different TGSRs that were inversly related to their plasma cholesterol. The fact that the TGSRs obtained by others in squirrel monkeys fed cholesterol (7) and in normocholesterolemic rhesus monkeys (15,16) are similar to what would be predicted from their cholesterol values when using our equation (Fig. 2) strengthens the inverse association between TGSR and the plasma cholesterol concentration, and suggests that the curve is applicable for cholesterolemias observed under a variety of dietary means.

The higher TGSR in the unsaturated fat-fed monkeys might be partially explained by increased percentages of triglyceride in VLDL (relative to protein, cholesteryl ester, and phospholipid) and slightly larger VLDL particles secreted by the liver in this group of monkeys (Hojnacki et al., in press), but this explanation does not account for the variation in TGSR within a dietary group.

In this regard, reexamination of recently published (6) and unpublished data in gerbils and rats fed different dietary fats has revealed a significant negative correlation be-

tween plasma cholesterol and TGSR when TGSRs were compared as a function of individual plasma cholesterol concentrations rather than by diet ($r = -0.68$; $P < 0.01$ for 13 gerbils and $r = -0.65$; $0.05 < p < 0.10$ for 9 rats). These data support the hypothesis that high concentrations of plasma cholesterol transported as the "product" lipoprotein, LDL, may inhibit the secretion of its "precursor" lipoprotein, VLDL.

One might argue that it is presumptuous to assume that the fasting TGSR represents the 24-hr secretion rate since in the fed animal the contribution of chylomicrons and VLDL from the gut might alter, even reverse, the dietary fat effect observed during fasting. This is conceivable when dietary fats differ, but it is again important to recall that the TGSR varied within diet as well as between diets and was best correlated inversely with the circulating cholesterol concentration. Even when the type of dietary fat differed, the total amount of fat digested and absorbed by the intestine in the two groups was essentially identical, so ultimately (each 24 hr) the total contribution from the gut should be identical. However, differences in intestinal packaging of lipid (chylomicrons vs. VLDL) due to the type of fat ingested or genetic variation within monkeys and the associated differences in rates of absorption are sufficient reasons to explore this possibility.

While several investigators have demonstrated that the addition of cholesterol to experimental diets results in a depression of hepatic cholesterol synthesis (20,21), little information is available concerning the influence of exogenous cholesterol on the synthesis/secretion of cholesterol containing lipoproteins. In fact, the reduction in VLDL triglyceride secretion rates in hypercholesterolemic monkeys reported here and elsewhere (7) and in similar studies in gerbils (6) are contrary to the in vitro findings in rats (22,23) where isolated, perfused livers from cholesterol fed rats had enhanced lipoprotein secretion rates. In those studies there was no correlation found between hepatic synthesis of various lipoprotein components and the secretion rate of the individual lipoproteins. The factor(s) that regulate lipoprotein secretion remain obscure although a recent report demonstrated that VLDL secretion was diminished in isolated rat livers perfused with glycerol (24). In view of these findings, the possibility exists that factors other than glycerol may also affect lipoprotein secretion such as a component of low density lipoproteins as suggested by our in vivo studies.

These results in monkeys and those in gerbils (6,7) suggest that elevations of LDL during hypercholesterolemia may occur by mechanisms other than overproduction of its precursor VLDL. We previously have postulated that the observed increase in plasma VLDL in gerbils fed coconut oil (6) and in certain monkeys fed the same diet for prolonged periods from birth (unpublished observations) may result from delayed clearance of lipoproteins. Low density lipoprotein turnover studies in squirrel monkeys (25,26) and our own cholesterol turnover studies in monkeys (27) tend to support this hypothesis since they collectively indicate that dietary induced hypercholesterolemia is associated with reduced fractional catabolism of lipoprotein lipid and protein.

Assuming that all Tritonized animals in this study were in a steady state condition, i.e., the secretion of lipoprotein equaled clearance, the data suggest that monkeys fed the saturated fat diets not only had reduced VLDL secretion but impaired VLDL clearance as well. This conclusion is compatible with the reduced lipoprotein lipase activity associated with the feeding of saturated fat diets (28,29). In addition, lipoproteins secreted at a faster rate, as in our animals fed the unsaturated fat diets, tend to be larger (30), and should be cleared faster since larger chylomicron and VLDL particles are preferentially catabolized by lipoprotein lipase (31,32). Our recent observation that monkeys fed corn oil have larger particles than those fed coconut oil is further supporting evidence (33).

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A Soy Extract Catalyzes Formation of 9-Oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic Acid from 13-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic Acid[1]

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ABSTRACT

In the presence of oxygen, a crude soy extract converted 13-hydroperoxy-*cis*-9, *trans*-11-octadecadienoic acid into numerous products, from which 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid was isolated. Additionally, the soy extract oxidized linoleic acid to the oxo-epoxyoctadecenoic acid, presumably via a sequential reaction involving lipoxygenase oxidation of linoleic acid followed by degradation of the resultant linoleic acid hydroperoxide. However, the linoleic acid substrate yielded two isomeric linoleic acid hydroperoxides and because of this, two isomeric oxoepoxyoctadecenoic acids.

INTRODUCTION

Blain and Styles (1) observed a linoleic acid hydroperoxide (LOOH) degradation in buffered extracts of defatted soybeans. Subsequent work (2,3) showed that the rate of hydroperoxide decomposition was decreased by dilution of the soy extract, and that the causative agent was heat labile. More recently it was demonstrated that soy lipoxygenase degraded the product of its own reaction, LOOH, to a variety of fatty acids under both aerobiosis (4-6) and anaerobiosis (7,8). Many of these fatty acids from LOOH degradation by soy lipoxygenase are similar to those obtained by homolysis of LOOH by a chemical model (9).

Evidence has been lacking to show that soybeans have a specific enzyme to decompose LOOH. LOOH-isomerase is an enzyme found in most cereals as well as in other plants (10). In the present study, we used a crude soy extract so that the presence of a LOOH-isomerase might be detected if present. Instead, we found a large variety of products among which were those fatty acids obtained by degrading LOOH by the chemical model. In particular, oxo-epoxyoctadecenoic acid, extant in the chemical model, was isolated as a product of soy extracts for the first time.

[1]Presented in part at the 13th Congress, International Society for Fat Research, Marseilles, France, August 30-September 4, 1976.

METHODS

Preparation of Soy Extracts

A crude soy homogenate was prepared by thoroughly grinding 10 g whole soybeans (Burpee Kanrich, 1975 crop) with 100 ml 0.1 M phosphate buffer (pH 6.9). During grinding with a mortar and pestle, the homogenate was kept cold with an ice bath. The homogenate was strained through cheesecloth, and then was centrifuged at 8000 x g for 15 min. The top layer of spherosomes (oil droplets) was carefully removed from the centrifugate yielding a clear supernatant, which was the crude extract used in this study. The extract was kept under N_2 at 0 C before use.

Reaction Conditions

In one reaction, the soy extract catalyzed the decomposition of 99+% 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoic acid (13-LOOH). The 13-LOOH (300 mg), prepared according to Gardner (11), was emulsified in 60 ml 0.05 M phosphate buffer (pH 6.9) containing 0.5% Tween 20. This substrate solution and the soy extract (44 ml) were briefly oxygenated with pure oxygen before mixing. Thorough oxygenation continued after mixing by bubbling oxygen into the solution through a fritted glass disc. After 20 min reaction time at 21 C, the solution was adjusted to pH 4 with HCl and immediately extracted with 400 ml $CHCl_3$:CH_3OH 2:1.

In another reaction, the substrate was 99+% linoleic acid (NuChek Prep, Elysian, MN). All concentrations and conditions were the same, except the reaction time was 30 min.

Chromatography and Analytical

Initially, the products were separated by a hexane-ether elution through a column packed with Silic-AR CC-4 as described previously (9). The compound of interest, oxoepoxyoctadecenoic acid, was isolated after triple development by thin layer chromatography (TLC) on a 250 μ Silica Gel G plate with hexane-ether-acetic acid, 70:30:1. Alternatively, the oxo-epoxyoctadecenoic acid was methyl esterified and separated by TLC with hexane-ether, 50:50.

TABLE I

Column Chromatography of Products from Degradation of 13-Hydroperoxy-*cis*-9,*trans*-11-octadecadienoic Acid (13-LOOH) by Soy Extract

Elution volume (liter)	Wt %[a]	Identity[b]
0.14-0.30	3.4	Aldehydes[c] and other
0.31-0.57	37.2	Unreacted hydroperoxide and 13-oxooctadeca-9,11-dienoic acid
0.58-0.64	2.3	9-oxo-12,13-epoxyoctadec-9-enoic acid and 13-hydroxyoctadeca-9,11-dienoic acid
0.65-1.41	38.4	Numerous compounds including 11-hydroxy-12,13-epoxyoctadec-9-enoic acid

[a]Wt percent was based on 300 mg 13-LOOH used as substrate.

[b]Fatty acids were identified after TLC isolation by comparison of their spectra (NMR, MS) with those identified previously (9).

[c]The aldehydes were weighed as their 2,4-dinitrophenylhydrazones.

Gas liquid chromatography (GLC) was utilized in tandem with a mass spectrometer (12). One GLC column (6 ft x 4 mm) was packed with 3% Silar 5CP on Gas-Chrom Q. The column temperature was programmed from 175-245 C at 5 C/min to separate methyl 9-trimethylsilyloxy-12,13-epoxy-10-octadecenoate. Another column (4 ft x 4 mm), used for separation of methyl 9-oxo-13-trimethylsilyloxy-12-methoxy-10-octadecenoate, was packed with 3% OV-1 on Gas-Chrom Q. The column temperature was programmed from 200 to 250 C at 4 C/min.

Spectral analyses and chemical transformations were accomplished as described previously (9). Epoxides were opened with 10% BF_3 in methanol held at 52 C for 6 min.

RESULTS AND DISCUSSION

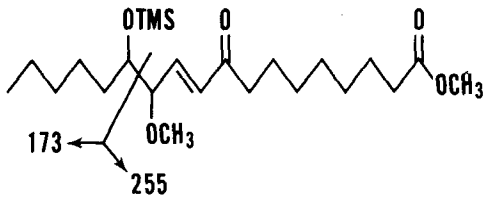
A crude soy extract catalyzed the decomposition of 13-LOOH to a large number of products (Table I).

Among the products was 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid (I), which was identified as a product from soy for the first time. Nuclear magnetic resonance (NMR), infrared (IR), and ultraviolet (UV) spectra of isolated I were identical to those reported before (9). Together, the spectral data showed that I was a fatty acid containing a *trans*-epoxide and a keto-carbonyl separated by a *trans*-double bond.

In order to locate the positions of the functional moieties of I, mass spectroscopy (MS) was employed. I (methyl ester) yielded weak fragment ions, but another derivative, methyl 9-trimethylsilyloxy-12,13-epoxyoctadec-10-enoate, prepared by $NaBH_4$ reduction of I (methyl ester) followed by silylation with hexamethyldisilazane:trimethylchlorosilane:pyridine, yielded a spectrum nearly identical to the one reported for methyl 9-trimethylsilyloxy-12,13-epoxy-octadec-10-enoate by Hamberg (13). Because a relatively large 173 m/e ion of unknown origin confused the isomeric analysis, a derivative was formed by treating I sequentially with BF_3-methanol and hexamethyldisilazane:trimethylchlorosilane:pyridine (Table II). The MS data indicated that the BF_3-methanol treatment of the epoxide resulted in a ring opening with most of the methoxy being substituted at carbon-12 (256, 328 m/e). Because C-12 is allylic, it can sustain

TABLE II

Mass Spectrum of a Derivative[a] of 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic Acid



Ion (m/e)	Possible origin	Relative intensity (%)
73	TMS [$Si(CH_3)_3$]	92.5
103		19.6
173		98.2
224	255 - 31	3.4
256	255 + 1	13.7
328	255 + 73R[b]	100.0
382	M-(31 + 15)	0.9
413	M-15	1.1

[a]Methyl 9 - oxo-13-trimethylsilyloxy-12-methoxy-*trans*-10-octadecenoate was obtained by BF_3-CH_3OH treatment of 9-oxo-*trans*-12,13-epoxy-*trans*-10-octadecenoic acid.

[b]73R results from migration of the trimethylsilyl (TMS) group from carbon-13 to a carbonyl (12).

TABLE III

Column Chromatography of Products from Oxidation of Linoleic Acid by a Soy Extract

Elution volume (liter)	Wt (%)[a]	Identity[b]
0.14-0.33	3.0	---
0.34-0.56	17.2	Linoleic acid hydroperoxide and oxooctadecadienoic acid
0.57-0.65	9.9	Mostly oxoepoxyoctadecenoic acid
0.66-1.40	50.5	Numerous compounds

[a]Wt percent based on 450 mg linoleic acid used as substrate.
[b]See footnote b, Table I.

a positive charge more readily under acidic conditions leading to methoxy-substitution at C-12. The 173 m/e ion was diagnostic of a 13-trimethylsilyloxy group, and this ion confirmed that the derivative was methyl 9-oxo-13-trimethylsilyloxy-12-methoxy-10-octadecenoate.

It is noteworthy that little evidence was found in the above MS for the existence of another possible isomer, i.e., methyl 13-oxo-9-trimethylsilyloxy-10-methoxy-*trans*-11-octadecenoate. Our unpublished results with the latter compound show particularly intense fragment ions at 170 (169+1), 242 (169+73), and 259 m/e. The MS reported in Table II had very weak ions at the following m/e: 170 [1.1% relative intensity (RI)], 242 (2.4% RI), and 259 (1.5% RI).

In addition to I, other fatty acids were identified in the product mixture, Table I. These fatty acids were reported by others (4-8) to result from decomposition of 13-LOOH by soy lipoxygenase. A considerable number of other compounds were not identified because of the complexity of the mixture.

Soy extracts can also catalyze production of I from linoleic acid. A sequential reaction starting with lipoxygenase-oxidation of linoleic acid to LOOH and ending with decomposition of LOOH gave the results shown in Table III. Except for a trace, linoleic acid was nearly completely oxidized. The products appeared to be similar to those obtained from the decomposition of 13-LOOH, including formation of a significant quantity of I. Although the reaction with linoleic acid was only 10 min longer than with 13-LOOH, the yield of I with linoleic acid was much improved.

GC-MS analysis showed that I derived from linoleic acid was mixed with a smaller quantity of 13-oxo-9,10-epoxy-11-octadecenoic acid. For GC-MS, the mixture was converted to the appropriate derivative as in Table II. In the MS the two most intense ions, assigned to methyl 9-oxo-13-trimethylsilyloxy-12-methoxy-10-oxtadecenoate (173 and 328 m/e), were 6 to 7 times as intense as the corresponding ions assigned to methyl 13-oxo-9-trimethylsilyloxy-10-methoxy-11-octadecenoate (259 and 242 m/e). Therefore, I is predominant in the mixture. This result is consistent with the formation of the two compounds from a mixture consisting of mostly 13-LOOH with smaller amounts of 9-hydroperoxyoctadeca-10,12-dienoic acid. Soy lipoxygeanse is known to catalyze the oxidation of linoleic acid to such a mixture of hydroperoxides (10).

The production of I from 13-LOOH was common to both soy extracts and a chemical model employing ionic iron and cysteine (9). With the chemical model, Gardner (10) proposed a pathway for formation of I. In this pathway, ferrous ion catalyzed homolytic cleavage of the hydroperoxy group yielding an alkoxy radical. The alkoxy radical could then form an epoxy ring by attack of the olefinic carbon α to the carbon bearing the alkoxy-oxygen radical. The keto group at carbon-9 undoubtedly is derived from molecular oxygen via a hydroperoxy group.

The relationship of soy extract products with those from the chemical model extends beyond the formation of I. Products common to the model and soy systems are oxooctadecadienoic acid (7), 11-hydroxy-*trans*-12,13-epoxy-*cis*-9-octadecenoic acid (6), trihydroxyoctadecenoic acid (5), 9(13)-oxo-13(9)-hydroxyoctadec-11(10)-enoic acid (14), and hydroxyoctadecadienoic acid (5). Thus, it appears that LOOH is degraded by a similar mechanism in both chemical and soy extract systems. The reactions are probably initiated by homolysis of the hydroperoxy group to an alkoxy radical. Soy lipoxygenase alone has been observed to catalyze production of the above fatty acids. The active center of lipoxygenase contains iron (15), and possibly a nonspecific action of a ferro-protein may be the factor causing hydroperoxide homolysis. In the crude soy extract, other ferro-proteins, such as

peroxidase and cytochromes, are present. Undoubtedly, their additive effect is responsible for this nonspecific degradation of LOOH in soy. With the exception of I, Galliard et al. (16) found many of the same fatty acids in potato extracts, thus implicating a more widespread occurrence of this nonspecific hydroperoxide-degrading system in plants. However, one cannot generalize that soy does not contain an enzyme specific for hydroperoxides; to emphasize this point, the potato extract yields an enzymically produced divinyl ether at pH values above 7.

Further work with the chemical model is showing even further analogies to the soy systems and will be the subject of a future communication.

ACKNOWLEDGMENT

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Spectrofluorescent Detection of Malonaldehyde as a Measure of Lipid Free Radical Damage in Response to Ethanol Potentiation of Spinal Cord Trauma

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ABSTRACT

Studies of the role of free radical damage to the spinal cord following a 400 g-cm impact have suggested an increase in at least one free radical product, malonaldehyde, 24-36 hr post injury. To investigate further the role of free radical lipid peroxidation in degeneration of the spinal cord following injury, a study of specific lipid fluorescence (SLF) indicative of the double Schiff-base adduct formed by a reaction between malonaldehyde and cellular components was carried out in the presence of ethanol, a known potentiator of free radical lipid peroxidation. The study was carried out in cats who received a 200 g-cm impact 3 hr to 23 days prior to sacrifice. Half of the impacted animals received ethanol, 5 ml/kg, prior to injury. These animals were rendered paraplegic, whereas the non-ethanol treated animals were neurologically intact. Controls consisting of laminectomies alone or laminectomies with ethanol but without injury were also studied. Spinal cord segments at the impact or laminectomy site were minced and extracted with chloroform-methanol, cleared by centrifugation, and examined in a scanning fluorometer with excitation maximum at 360 nm and emission maxima at 420, 440, 450, and 460 nm. SLF was minimal in cats 3 hr and 1 day post injury, but markedly increased at 3 days. By 5 days, background levels were again found in all groups. SLF in the alcohol-pretreated impact animals rose to a peak at 7 days, followed by a decline to background by 10 days. The presence of SLF supports a role for free radical lipid peroxidation in the degenerative changes in the spinal cord following injury. The findings of two peaks of SLF activity suggest two different sites of damage. One site, found acutely after injury, appears in all groups and was associated with reversible changes, while the other site is associated with later changes and chronic paraplegia only. The two sites could be the gray and white matter.

Pathologic free radical reactions among susceptible membrane lipids of the central nervous system following trauma have been suggested as a molecular basis for injuries to the central nervous system (1-3). Malonaldehyde, a product of free radical degradation of unsaturated fatty acids, has been shown to be elevated in spinal cords of cats injured with a 400 g-cm impact (4), as well as in freeze-lesioned rat brains (5). Other evidence suggesting that radical reactions may be involved in central nervous system injury include the consumption of endogenous tissue antioxidants following similar spinal cord injuries (3), the reduction of cerebral edema following cold injury by the administration of an exogenous antioxidant (6), reduction in malonaldehyde levels in freeze-lesioned rat brains with corticosteroid pretreatment (5), and the loss of extractable cholesterol from the central nervous system following cold injury (7).

A more recent model of spinal cord injury has been developed in this laboratory (8). It utilizes a 200 g-cm impact to the spinal cord of cats in conjunction with the systemic administration of ethanol, an agent known to enhance lipid free radical reactions. Animals injured with a 200 g-cm impact alone regain neurologic function, while those injured after the administration of ethanol remain permanently paraplegic. The present study was directed to the question of whether this physiochemical model of paraplegia might involve free radical mechanisms that could be measured.

Malonaldehyde is well known as a by-product of free radical peroxidation of poly-unsaturated fatty acids (9). The most common method for measuring this 3 carbon dialdehyde utilizes its reaction with 2-thiobarbituric acid (TBA). Malonaldehyde, as produced, is not stable but a reactive molecule which is readily metabolized. It can also form cross-links with

TABLE I

Spinal Cord Injury – TBA Assay

Procedure	Time of sacrifice				
	0 hr	3 hr	5 hr	24 hr	Total
Laminectomy	4	2	2	2	10
Ethanol-laminectomy[a]	--	2	1	1	4
200 g-cm impact	--	4	3	3	10
Ethanol-200 g-cm impact[a]	–	4	3	3	10
Total	4	12	9	9	34

[a]Ethanol administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 100 ml, as a constant infusion for 1 hr prior to laminectomy or injury.

TABLE II

Spinal Cord Injury – Fluorescence

Procedure	Time of sacrifice							
	3 hr	24 hr	3 days	5 days	7 days	10 days	23 days	Total
Laminectomy[a]	4	4	7	6	9	8	4	42
200 g-cm impact	4	4	7	6	9	6	4	40
Ethanol-200 g-cm impact[b]	4	4	7	6	9	6	4	40
Total	12	12	21	18	27	20	12	122

[a]Laminectomy-ethanol animals (6) were sacrificed at critical times (3 hr, 3 days, 5 days) and found to have similar values to laminectomy alone. Thereafter all laminectomies were performed without prior infusion with ethanol.

[b]Ethanol administered as an infusion of 95% ethanol, 5 ml/kg, diluted with saline to a volume of 100 ml, as a constant infusion for 1 hr prior to laminectomy or injury.

amino-containing phospholipids, proteins, and nucleic acids (10). However, with these reactions, it becomes undetectable by the TBA test. These cross-links are conjugated double Schiff's base adducts and are fluorescent. They can be measured more reliably as an indicator of malonaldehyde production (10). In an attempt to avoid the inconsistencies observed with the TBA analysis, we have chosen to measure lipid soluble fluorescence (LSF) as an indicator of free radical reaction (11). In the present study, lipid soluble fluorescence and TBA-reactive material were measured in extracts of the traumatized spinal cords of cats which received or did not receive ethanol.

MATERIALS AND METHODS

Spinal Cord Injury

One hundred and fifty-six cats were used for the spinal cord injury study, 34 in the TBA study and 122 in the LSF study. All animals were anesthetized with sodium pentobarbital, 30 mg/kg. They were allowed to breathe spontaneously. Body temperature was maintained with a heating blanket. Arterial blood pressure and blood gases were monitored (8). Blood alcohol levels were measured spectrophotometrically with a dichromate assay (8).

Spinal cord injury was produced according to a method previously reported from this laboratory (8). A laminectomy was carried out from T7 through T11. A 20 g weight was dropped 10 cm down a Teflon tube to strike an impactor, thereby delivering a 200 g-cm force injury. Cortical evoked potentials were monitored prior to laminectomy, after laminectomy, and for 3 hr after injury.

A laminectomy group and a 200 g-cm injury group received 95% ethanol (5 ml/kg) diluted with saline to a volume of 30 ml as a constant infusion for 1 hour prior to laminectomy or injury. Fifty-two animals underwent laminectomy alone, four received ethanol prior to laminectomy alone; 50 animals received ethanol followed by a 200 g-cm impact; and 50 animals received a 200 g-cm impact without ethanol. Table I shows the distribution of the 34 cats used in the TBA assay study, and Table II shows the distribution of the 122 cats used in the LSF study, as well as the times of sacrifice of the different animals. After sacrifice, a 3 cm segment of spinal cord, centered over the site of injury, was removed for fluorescent analysis and TBA assay

TBA Assay

About 10% of the malonaldehyde found is free and produced following the oxidation of polyenoic fatty acids by double β-scission. This produces the 3-carbon dialdehyde and two hydrocarbon radical fragments. However, 90% of the oxidized fatty acids are meta-stable in the form of hydroperoxy- and cyclic endoperoxides (9). These meta-stable products are cleaved under the strongly acid conditions of the assay to form free malonaldehyde which is detected by the production of a chromogen, formed by reaction with two molecules of 2-thiobarbituric acid (TBA)(9). For the TBA assay, a weighed sample of spinal cord (about 0.4 g) was minced and homogenized in 5 ml of a mixture made up as follows: 10 ml of a saturated TBA solution in 10% perchloric acid (PCA) added to 30 ml of 20% trichloroacetic acid (TCA). The resulting homogenate was heated in a boiling water bath for 20 min and then centrifuged for 15 min at 2500 rpm. A 3 ml aliquot of the supernatant was removed and read at 532 nm on a Beckman DU spectrophotometer against a reagent blank.

Lipid Soluble Fluorescence

Spinal cord segments (1 g) were minced with two scalpel blades to less than 125 mm^3 pieces in 10 ml of carbonyl-free fluorescent grade chloroform and methanol (2:1, v/v) for several minutes, mixed with an equal volume of deionized water, and centrifuged for 5 min at 2500 rpm. One milliliter of the chloroform layer was mixed with 0.1 ml of methanol and exposed to high intensity ultraviolet light for 1 min (11). Fluorescence intensity was measured from excitation (360 nm) and emission (440 nm) spectra obtained at 25 C on a Hitachi-Perkin Elmer MPF 3 spectrofluorometer in the direct mode standardized with quinine sulfate (11). The measured wavelength was not corrected for internal instrument distortion. Excitation and emission slits were set at 8 nm; the sensitivity was set at 30. Fluorescence was recorded and expressed in arbitrary units based on these instrument settings. Typical fluorescent excitation and emission spectra are represented in Figure 1 (11). Fluorescence variation was generally not more than 20%.

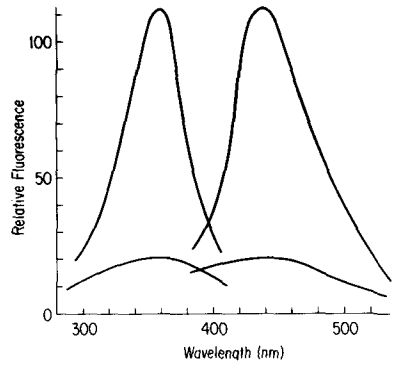

FIG. 1. Fluorescence excitation and emission spectra of chloroform:isopropanol extracts of tissue lipids. Maxima were 360 nm for excitation and 440 nm for emission. Lower curves were obtained from control tissue and show no distinct peaks, while upper curves are from a damaged tissue sample.

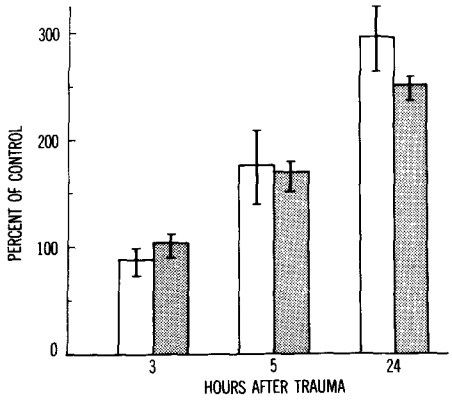

FIG. 2. Effects of alcohol and trauma on malonaldehyde levels in cat spinal cord. The ordinate represents a comparison of the means as a percent of the control (laminectomy alone). Vertical bars are the standard error of the mean (SEM). Although there was no statistical difference at any one time interval between the impacts and alcohol-impacts, there did appear to be significant differences between time intervals (see Table II). Open bars represent the 200 g-cm impact alone, closed bars represent alcohol-impacts. There are four animals in each 3 hr group, six in the 5 hr group, and six in each 24 hr group.

RESULTS

Spinal Cord Injury

During the 3-hr monitoring period, all 35 cats in the alcohol-laminectomy group retained their evoked potentials and were without neurological deficit after surgery. Those followed up to 23 days showed no neurological deterioration. The 37 animals in the 200 g-cm group had all regained their evoked potentials by the end of the 3-hr monitoring period and were walking by 7 days. The 37 animals in the alcohol-impact group lost their evoked potentials and did not regain them within the 3-hr monitoring period. These animals remained paraplegic until sacrifice (Table I).

TABLE III

Effects of Alcohol and Trauma on Malonaldehyde Levels in Cat Spinal Cord

Time (hours)	Malonaldehyde level[a] nmol/g	% of control	Significance (degrees of freedom, t value, P value)
3	31.2 ± 2.7	95.6 ± 8.4	
			t_{10}, 4.04, <.01
5	272.4 ± 27.1	173.0 ± 17.2	
			t_{10}, 4.08, <.01
24	340.2 ± 21.8	273.5 ± 17.5	

[a]Malonaldehyde levels by the TBA method were obtained by spectrophotometry and converted to nanomoles per g wet weight of tissue. The data were then converted to percent of laminectomy control for tabulation. Statistical analysis was performed using the Student's t-test, a comparison of the means. Confidence levels were chosen according to the two-tailed Z method. Since the impact groups and alcohol-impact groups showed no significant differences (Fig. 2), the two groups were pooled for each time and the combined groups compared against time. Since 5 hr animals were significantly higher than the 3 hr group, and the 24 hr animals also significantly higher than the 5 hr group, it follows that the 24 hr group was very significantly higher than the 3 hr group.

Malonaldehyde—Direct

At 3 hr, no differences in TBA reactive materials were observed between the impact group or the alcohol-impact group as compared to the controls (Fig. 2). At 5 and 24 hr, impacts and alcohol-impacts were two and three times the controls, respectively ($p<0.01$). Data from both groups were pooled for each time interval to perform Student's t-test for comparison of the means (Table III).

Malonaldehyde—Indirect

No differences were observed in the levels of lipid soluble fluorescence among the three groups when measured at 3 and 24 hr following sacrifice. A threefold increase was observed among all three groups at 3 days. By 5 days, this returned to background. A second elevation was observed at 7 days in the alcohol-impact group, but not in the laminectomy-ethanol group or the impact group. At 10 days and 23 days, lipid soluble fluorescence in all three groups had returned to background. These results are summarized in Figure 3.

Blood pressure and arterial blood gases did not vary significantly from baseline levels in all groups. The infusion rate of alcohol was adjusted to insure that bradycardia and hypotension did not occur. A mean blood level of ethanol of 350 mg % was obtained in the ethanol infusion groups. In the noninfused group, a blood level of 30 mg % ethanol was observed due to the ethanol contained in the commercial sodium pentobarbital preparation.

DISCUSSION

In the cat, a 400 g-cm impact to the exposed spinal cord alone invariably produces paraplegia (8). On the other hand, a 200 g-cm force produces a reversible lesion, unless the animals have been pretreated with ethanol to produce blood levels of 300-400 mg %, in which case, the same 200 g-cm force produces irreversible paraplegia (8). Malonaldehyde, a product of free radical peroxidation of lipids was measured in two ways: directly by the TBA assay and indirectly by lipid soluble fluorescence (LSF).

Malonaldehyde levels measured by the TBA assay rose in all three groups at 5 and 24 hr. However, no differences could be detected between impacted animals and animals pretreated with alcohol before impaction utilizing this assay (Fig. 2). TBA reactive material was not measured beyond the 24-hr period. LSF rose to similar levels above background at 3 days in all groups. By 5 days, LSF had returned to background in all groups. At 7 days, a second peak of LSF activity had occurred in the alcohol-impact group only. Only background LSF activity was noted after 7 days.

The source of malonaldehyde detected by both methods is most likely the polyunsaturated fatty acids of membrane phopholipids. Malonaldehyde cannot be produced as a consequence of radical damage to cholesterol or saturated fatty acids alone (12). Polyunsaturated fatty acids are needed (9). Phospholipids, as they occur in myelin and other cellular membranes, represent the largest source of unsaturated fatty acids in the central nervous system (13).

While LSF develops slowly and accumulates in detectable quantites only after several days, it is not entirely stable, since background levels are again achieved within 2 days after peak levels are produced. The induction period prior

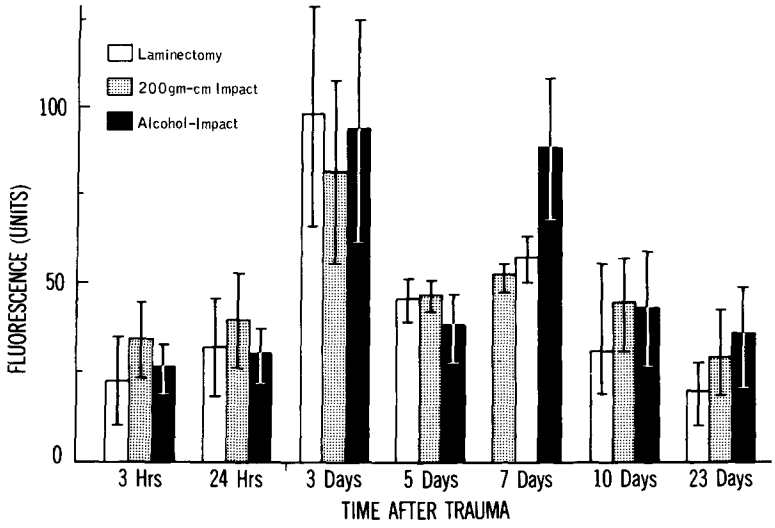

FIG. 3. Effects of alcohol on levels of lipid soluble fluorescence in traumatized spinal cord. The ordinate represents arbitrary fluorescence units taken from the photomultiplier reading and normalized to a constant setting. The open bars represent the laminectomy group, the stippled bars represent the 200 g-cm impact group alone, and the closed bars represent the alcohol-impact group. The vertical bars represent the SEM.

to the first fluorescence peak is due to single Schiff's base adducts which form rapidly from malonaldehyde in the presence of free amino groups. These do not contain conjugated double bonds and are not fluorescent (latent fluorescence) (10). Only when the second adduct is formed as a cross-link does the malonaldehyde product become fluorescent (expressed fluorescence) (10).

Although 1-2 days may be required for latent fluorescence from malonaldehyde to be expressed, once formed, it will be expressed (11). Of note, a second LSF peak occurs at 7 days, but only in the alcohol-impact group which developed paraplegia. Because of the kinetics of latent fluorescence, i.e., formation of the conjugated cross-link, this second peak is indicative of a second wave of malonaldehyde production (10). This suggests that the first peak of fluorescence, seen in all groups, is associated with acute, reversible damage related to the surgery and trauma, whereas the second peak is associated only with irreversible degeneration within the spinal cord.

The data are compatible with the hypothesis that free radical mechanisms are involved in the alcohol-impact injury model. Possibly the added burden of free radical reactions caused by the presence of alcohol depletes aqueous antioxidants such as ascorbate (3) to levels which allow irreversible membrane lipid damage. The two peaks of LSF activity seem to reflect two separate sources or pools of unsaturated fatty acids. The former one, which is rapidly oxidized, is not associated with any noticeable permanent deficit. The latter source, one which oxidizes at a slower rate (because of lower levels of polyunsaturates and a larger lipid-soluble antioxidant pool), is correlated with irreversible damage. The two different sources, for example, could be the central gray matter and surrounding long tracts.

The central gray area, whose phospholipids are largely polyunsaturated, would undergo peroxidation more easily as a result of minimal surgical trauma, such as laminectomy alone and impact alone. Necrosis of a thoracic segment of central gray tissue, for example, while producing local loss of some neuronal elements, produces no grossly discernible sequella like paraplegia. An early peak in malonaldehyde levels would be reached in both surgically treated groups of animals irrespective of alcohol pretreatment. Since a full complement of protective enzymes (e.g., superoxide dismutases, catalases, peroxidases, glutathione reductase and oxidase, ascorbate oxidase) exist in all actively metabolic cells of the CNS, the biochemical debris would be rapidly delimited and removed (14), although any physiological loss of function would not be reestablished. The early peak of malonaldehyde in the laminectomy and alcohol-laminectomy controls (by either TBA or LSF) would have to be the result of damage at the subcellular level, since histologically there is no discernible damage to central gray or white matter in these animals.

Quite the opposite is true of the white

matter in long tracts where edema accumulates. The kinetics of peroxidation in this highly saturated, cholesterol-rich (75% of total CNS cholesterol is found in the myelin) tissue is very slow. However, the myelin is largely metabolically inert. There are essentially no enzyme systems to bear the brunt of a peroxidative attack (14). Once the cholesterol has been oxidized to any appreciable extent, membrane integrity is quickly destroyed (15). Damage in this tissue would be irreversible.

Cholesterol in the plasma membranes of most cells represents a pool which is rapidly exchangeable with free plasma cholesterol (16). The opposite case exists for cholesterol contained in lipid droplets, organelle membranes within the cell, or the multilaminar extruded plasma membranes of the myelin sheath. Exchange rates of the order of 10% of total or less in 7 hr is a typical figure for lipid droplets and subcellular organelle membranes (16). Myelin, because of its multilayered configuration, and isolation from the general circulation, represents a terminal storage site for cholesterol (16). There are no mechanisms for removal of any damaged steroids from this site, other than by simple diffusion out of the membrane (16).

The data are generally supportive of the hypothesis that free radical mechanisms are operative during traumatic injury to spinal cord. Of even greater importance are the findings which suggest two major sites of damage in such injuries: one rapid and reversible, the other slow but permanent. If the two sites responsible for the production of malonaldehyde are, in fact, the central gray and surrounding white matter, then this represents a critically important concept for all of the CNS since, in any potentially reversible condition, it will be the white matter integrity which defines the limits of permanent damage.

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The Effect of Dietary Fatty Acid Balance on Myocardial Lesions in Male Rats[1]

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ABSTRACT

Three hundred (experiment I) and 350 (experiment II) weanling, 3-week-old male Sprague-Dawley rats weighing between 40-50 g were randomly assigned two per cage and 50 per dietary treatment to study the effect of dietary fatty acid balance on myocardial lesions. The following oils were tested: Experiment I. *Brassica napus* var. Tower rapeseed oil [Tower RSO, 1974 cultivar and 1975 cultivar, each containing 0.3% erucic (22:1) acid]; *B. napus* var. Zephyr RSO containing 0.9% 22:1; corn oil; olive oil; and soybean oil. Experiment II. *B. napus* var. Tower RSO (1974 cultivar), olive oil, soybean oil, and the following oils to which was added the indicated level of free 22:1; Tower + 0.5% 22:1; Tower + 5.6% 22:1; olive oil + 4.4% 22:1; soybean oil + 5.7% 22:1. In each case the oils were incorporated in a semisynthetic diet at a level of 20% by weight. Heart and heart lipid weights of rats fed the different oils did not differ statistically from each other. Fatty acid analyses of heart lipids revealed that the fatty acid composition of the cardiac lipids reflected that of the diet fed. In experiment I, there was a definite but significantly lower incidence ($P<0.01$) and severity ($P<0.01$) of heart lesions in rats fed control oils (corn, olive, soybean) than in rats fed rapeseed oils. Also, in experiment II, a definite but lower incidence and severity of heart lesions occurred in rats fed control oils (soybean, olive) compared to rats fed Tower RSO or this oil with added free 22:1. Adding 22:1 to an oil naturally high in 18:3 (soybean) did not alter the incidence of heart lesions, whereas adding 22:1 to an oil naturally high in 18:1 (olive) increased significantly ($P<0.01$) both the incidence and severity of heart lesions. Thus, it appears that the background incidence of heart lesions that are found in the rat in any case, and which are increased by rapeseed oil feeding, is caused by the imbalanced fatty acid composition of the oil for the growing rat, i.e., high monoenes (18:1,20:1, and 22:1) and high 18:3 and is not only due to the presence of excess 18:3.

INTRODUCTION

Three suggestions have been made as to the cause of the long term myocardial lesions in male rats fed rapeseed oils. They are: (a) the erucic (22:1) acid content of the oil; (b) some specific toxic component present in the oil; and (c) fatty acid imbalance for the male rat.

Abdellatif and Vles (1) concluded from a series of experiments that the pathogenic characteristics of rapeseed oil could only be ascribed to its content of long chain fatty acid, especially erucic acid. This observation and conclusion were confirmed recently by Engfeldt and Brunius (2). In the latter study, rats fed Canadian LEAR oil (cultivar Oro) containing 0.3% 22:1 showed no evidence of myocardial necrosis. Furthermore, Vles et al. (3) found the incidence and severity of heart lesions to be the same in rats fed a Canadian LEAR oil (cultivar Tower), French LEAR oil (Primor) containing 0.3% erucic acid, or sunflowerseed oil. On the other hand, Hulan et al. (4) found that rats fed diets containing either commercial lard to which was added 5.4% free erucic acid or pig fat (RPF) containing 5.4% esterified erucic acid developed the same or lower incidence and severity of myocardial necrosis as rats fed commercial lard. They concluded that erucic acid per se is not responsible for the heart necrosis when rapeseed oils are fed to rats. In the case of Beare-Rogers et al. (5) and McCutcheon et al. (6), only when very high levels of erucic acid (30% or more) were added to nonrapeseed oils (i.e., olive oil, soybean oil) did an increase in cardiac lesions occur.

That some specific toxic component present in rapeseed oil causes the long term myocardial necrosis was suggested by Rocquelin et al. (7) and Beare-Rogers et al. (8). However, Kramer et al. (9) failed to remove any specific cardiotoxic factor from rapeseed oil by exhaustive molecular distillation and adsorption chromatography. From their studies, they concluded that the primary myocardiotoxic factor was the triglyc-

[1]Contribution No. 706, Animal Research Institute.

TABLE I

Fatty Acid Composition of the Diets

Fatty acids[b]	Diets[a]								
	Corn	Olive	Olive + 22:1	Soybean	Soybean + 22:1[e]	Tower[c]	Tower + 22:1	Tower + 22:1	Zephyr[d]
16:0	10.9	11.6	11.5	12.4	13.5	6.1	6.5	7.1	5.3
16:1	0.1	1.2	1.2	---	---	---	---	---	0.2
18:0	1.7	2.5	2.7	3.7	4.3	2.0	2.4	2.5	2.3
18:1	24.3	75.5	71.2	25.4	25.4	56.5	55.7	54.2	64.7
18:2	61.1	7.3	6.8	50.6	44.1	26.0	25.7	22.2	17.5
18:3	0.9	0.7	0.6	7.9	6.0	7.1	6.5	5.7	5.6
20:1	0.2	0.4	0.7	---	0.8	1.5	1.6	1.9	1.5
22:1	---	0.1	4.5	---	5.7	0.3	0.8	5.9	0.9

[a]Semisynthetic diets containing one of the vegetable oils listed at 20% by weight of the diet.

[b]Indicates number of carbon atoms:number of double bonds.

[c]Tower = *Brassica napus* var. Tower rapeseed oil. The fatty acid composition of the oil from the 1974 and 1975 cultivars of Tower was the same.

[d]Zephyr = *Brassica napus* var. Zephyr rapeseed oil.

[e]Free erucic acid (22:1) was blended into the vegetable oils as indicated. The source of erucic acid contained (mole %): 16:0-0.3; 18:0-0.5; 18:1-0.9; 20:0-1.3; 20:1-4.6; and 22:1-90.8.

erides of the oil (i.e., fatty acid composition and/or balance).

Hulan et al. (4) and Kramer et al. (9,10) suggested that the myocardial lesions in rats fed rapeseed oils result from a fatty acid imbalance not only from the presence of erucic (22:1) and eicosenoic (20:1) acids but also from altered saturate to unsaturate ratios, low concentrations of palmitic (16:0) acid, high concentrations of oleic (18:1) and linolenic (18:3) acids, and low 18:2/18:3 ratios. Additional evidence in support of this concept was presented by Hulan et al. (4) where it was demonstrated that the addition of erucic acid to an oil properly balanced in fatty acids for the rat, does not increase the incidence of myocardial lesions above background. McCutcheon et al. (6), on the other hand, concluded that the cardiopathogenesis of rapeseed oils did not appear to involve the balance (ratio) of linoleic and linolenic acid in the oil but rather the total linolenic acid content of the oil.

In light of the above, it was of interest to investigate the cardiopathogenicity of two different cultivars of LEAR-Tower (1974 and 1975 cultivars) and to investigate more thoroughly the effect of erucic acid and fatty acid balance on the incidence of myocardial necrosis in male rats.

MATERIALS AND METHODS

Two feeding trials (experiments) were conducted using male Sprague-Dawley rats. The following oils were tested: Experiment I. *Brassica napus* var. Tower rapeseed oil (Tower RSO, 1974 cultivar and 1975 cultivar, each containing 0.3% erucic acid); *B. napus* var. Zephyr RSO containing 0.9% erucic acid; corn oil; olive oil; and soybean oil. Experiment II. *B. napus* var. Tower RSO (1974 cultivar); olive oil; soybean oil and the following oils to which was added the indicated level of free erucic acid (22:1): Tower RSO + 0.5% 22:1; Tower RSO + 5.6% 22:1; olive + 4.4% 22:1; soybean oil + 5.7% 22:1.

Three hundred (experiment I) and 350 (experiment II) weanling 3-week-old male Sprague-Dawley rats weighing between 40-50 g were purchased (Bio-Breeding Laboratories, Ottawa) and randomly assigned 50 per dietary treatment. They were housed two per cage and fed ad libitum the test rations for 16 weeks. Water was available at all times. The semisynthetic diet, to which the test oils were added at a level of 20% by weight, have been described earlier (11). All rats were individually weighed at the beginning and at 2-week intervals throughout the experiment.

At the end of the experiment (16 weeks), five rats from each dietary group were killed by exsanguination under CO_2 anesthesia for heart lipid analyses. The hearts were immediately removed, weighed, frozen on dry ice, and pulverized. The frozen pulverized heart tissue powder was then placed in 25 ml $CHCl_3/CH_3OH$ (2:1) and homogenized at low speed using a Virtis 45 homogenizer. The homogenates were allowed to sit at room temperature for 1 hour, then filtered through a sintered glass funnel. Solvents were removed

TABLE II

Heart and Heart Lipid Weights and Fatty Acid Composition of the Heart Lipids of Male Rats Fed Experimental Diets for 16 Weeks (Experiment I)

	Diets[a]					
	Corn	Olive	Soybean	Tower RSO[b]	Tower RSO[c]	Zephyr RSO
Heart wt (g)	1.34[1 d]	1.25[1]	1.27[1]	1.16[1]	1.35[1]	1.39[1]
Lipid wt (mg/heart)	39.62[1]	40.26[1]	37.90[1]	33.10[1]	41.66[1]	37.52[1]
Fatty acids[e]						
16:0	11.91[1,2 f]	12.3[1]	11.5[1,2]	10.2[2]	12.9[1]	12.4[1]
18:0	24.2[2]	23.9[2]	25.6[1,2]	22.8[2]	30.0[1]	29.6[1]
18:1	9.7[2]	26.3[1]	10.0[2]	23.2[1]	18.9[1]	23.5[1]
18:2	27.2[1]	9.4	24.5[1]	21.0[2]	19.5[2]	16.0
18:3	-[g]	-	0.5[1,2]	0.7[1]	0.3[2]	0.2[2]
20:1	0.2[1,2]	0.3[1,2]	0.1[2]	0.4[1]	0.3[1]	0.2[1,2]
20:4	15.6[1,2]	17.8[1]	17.0[1]	13.8[2,3]	12.3[3]	10.9[3]
22:1	-	-	-	0.2[1]	0.1[1]	0.2[1]
22:4n-6	1.4	0.5[1]	0.7[1]	0.4[1,2]	0.2[2]	0.3[1,2]
22:5n-6	4.2	2.2	0.6[1]	0.1[1]	0.3[1]	0.3[1]
22:5n-3	-	0.1[2]	0.9[1]	0.8[1]	0.3[2]	0.3[2]
22:6n-3	1.8[3]	4.1[2,3]	6.2[1]	4.9[1,2]	3.6[2,3]	2.7[3]

[a]Semisynthetic diets containing one of the vegetable oils listed at 20% by weight of the diet.

[b]*Brassica napus* var. Tower rapeseed oil, 1974 cultivar.

[c]*Brassica napus* var. Tower rapeseed oil, 1975 cultivar.

[d]Means within a row lacking a superscript or without a common superscript are significantly different ($P<0.01$).

[e]Indicates the number of carbon atoms: number of double bonds; n represents the number of carbon atoms between the terminal double bond and the methyl end of the molecule. Small amounts of 14:0, 15:0, 17:0, 20:0, 16:1, 20:2, 20:3, and 22:0 were present as well as substantial quantities of dimethyl acetals of 16:0 (0.7-1.6%) and 18:0 (0.7-1.3%).

[f]Each value represents the mean of five rats.

[g]Not detectable.

using a rotary evaporator, dried under high vacuum, and total lipids were determined gravimetrically. Fatty acid analyses (experiment I) of an aliquot of the total lipids were carried out as described previously (10). The remaining rats from each dietary group were killed and the hearts examined histologically as described earlier (12).

Analyses of variance were determined for all methyl ester data (experiment I). The Duncan's New Multiple Rante Test (13) was used to determine significant differences ($P<0.01$) between treatment means. Approximate chi-square statistics, obtained following the approach of Fienberg (14), were used to examine the incidence and severity of myocardial lesion data for differences among the various dietary treatment groups of both experiments.

RESULTS AND DISCUSSION

The fatty acid composition of the diets is given in Table I. Compared to the diet containing corn oil, diets containing oils from the *Brassica* family were low in 16:0 and high in 18:1 and 18:3. The diets containing rapeseed oil also contained substantial quantities of 20:1 compared to the diets containing other vegetable oils. The rapeseed oil diets contained 22:1 of which the other vegetable oils (except olive oil) are completely devoid. It has been suggested that the cardiopathogenicity of rapeseed oil for the male rat is due to an imbalanced fatty acid composition of that animal, i.e., low saturates, low 18:2 to 18:3 ratio, high polyunsaturates (especially 18:3), and the presence of long chain monoenes (4,9,10) or the presence of high 18:3 (6). the diets used in experiment II of this study (Table I) were designed to test these hypotheses: high 18:1, low 18:3 (olive); high 18:1, low 18:3, presence of 22:1 (olive + 4.4% 22:1); high 18:3, no 20:1 or 22:1 (soybean); high 18:3 and the presence of 22:1 (soybean + 5.7% 22:1). These diet combinations also permitted the re-evaluation of the effect of free vs. esterified erucic acid in the presence of varying dietary fatty acid profiles.

The heart and heart lipid weights as well as the fatty acid composition of the heart lipids of rats fed in experiment I is presented in Table II. These data from experiment II will form the basis for a detailed study and discussion of various lipid subclasses of heart lipids of rats fed

TABLE III

Incidence and Severity of Myocardial Lesions in Male Rats: Experiment I

Diet[b]	Incidence[c]	Severity[a] 1-2	3-5	6-9	≥10
Corn	17/45	11	4	2	0
Olive	19/45	10	6	3	0
Soybean	19/45	12	4	3	0
Tower[d]	35/44	15	9	6	5
Tower[e]	34/45	18	9	6	1
Zephyr	36/45	12	15	2	7

Chi-Square Analysis[f]	d.f[g]	Chi-square
Complete experiment:	20	65.5***
among controls	6	1.1
among treated	8	11.3
treated vs. controls	4	53.0***
Incidence only:	5	40.0***
among controls	2	0.2
among treated	2	0.3
treated vs. controls	1	39.5***
Severity only:	15	25.5*
among controls	4	0.8
among treated	6	11.1
treated vs. controls	3	13.5**

[a]Severity = number of rats with lesion scores of 1-2, 3-5, 6-9, ≥10 per three sections of heart examined.

[b]Diet = semisynthetic diet containing one of the vegetable oils listed at 20% by weight.

[c]Incidence = number of rats affected with lesions out of the number of rats examined.

[d]Tower = *Brassica napus* var. Tower rapeseed oil, 1974 cultivar. One rat in this group died at 8 weeks.

[e]Tower = *Brassica napus* var. Tower rapeseed oil, 1975 cultivar.

[f]A comparison of rats affected to number of rats examined was used in the analysis of incidence. Only rats with heart lesions were compared in the analysis of severity. Significant differences * ($P<0.05$); ** ($P<0.01$); *** ($P<0.001$).

[g]The degrees of freedom (d.f.) of these analyses are (r-1) (c-1), where r and c are the number of rows and columns respectively. If an entire column was zero, the degrees of freedom were reduced accordingly.

rapeseed oils to be reported later.

As indicated in Table II, the heart weights and heart lipid weights of rats fed the different vegetable oils did not differ statistically from each other. The fatty acid composition of the heart lipids is also shown in this table. In general, the fatty acid content of the cardiac lipids reflected that of the diet fed. Heart lipids of rats fed rapeseed oils contained the same or a significantly ($P<0.01$) higher level of saturates (16:0, 18:0) than did the heart lipids of rats fed the other vegetable oils. On the other hand, cardiac lipids of rapeseed oil fed rats contained a similar level of 18:1 to the heart lipids of rats fed olive oil, similar level of 18:3 to the rats fed soybean oil, and lower levels of 18:2 and 20:4 than rats fed either corn oil or soybean oil. With but few exceptions, the levels of 20:1 in heart lipids were similar for all groups irrespective of oil fed. Not surprisingly, 22:1 was found only in the cardiac lipids of rats fed rapeseed oil. The higher levels of 22:4n-6 and 22:5n-6 found in heart lipids of rats fed corn oil than in rats fed the other oils arise from elongation and desaturation of the high levels of 18:2 ingested. Similarly, the higher levels of 22:5n-3 and 22:6n-3 in cardiac lipids of rats fed soybean oil or rapeseed oils resulted from the metabolism of high levels of 18:3. In general, the data presented in Table II are in good agreement with those already published for these oils (4,9,10,15,16).

The incidence and severity of myocardial lesions of the male rats fed in experiment I are given in Table III. As reported earlier (10) and confirmed by this laboratory (4,9,15-17) and others (2,3,6-8), there was a definite but lower incidence of myocardial lesions in rats fed control oils (corn, olive, soybean) compared to rats fed rapeseed oils. Chi-square analysis of these data (Table III) revealed that there were no significant differences in incidence or severity among rats fed control oils or among those fed rapeseed (treated) oils. On the other hand, rats fed rapeseed oil developed both a significantly higher incidence ($P<0.001$) and severity ($P<0.01$) of heart lesions than did rats fed the

TABLE IV

Incidence and severity of myocardial lesions in male rats: Experiment II

Diet[b]	Incidence[c]	Severity[a] 1-2	3-5	6-9	⩾10
Soybean	18/44[d]	14	3	1	0
Olive	13/45	12	1	0	0
Tower[e]	28/45	14	6	5	3
Tower + 0.5% 22:1	30/45	16	11	2	1
Tower + 5.6% 22:1	25/45	12	6	4	3
Soybean + 4.7% 22:1	19/45	16	1	0	2
Olive + 4.4% 22:1	27/45	16	7	2	2

Chi-Square Analysis[f]	Incidence d.f.[g]	Incidence Chi-square	Severity d.f.[g]	Severity Chi-square
Complete experiment	6	20.90*	18	29.56*
Between controls	1	1.4	2	1.8
Among treated	4	6.4	12	17.2
Controls vs treated	1	13.1***	3	10.6*
Olive vs Olive + 22:1	1	9.0***	3	13.44***
Soybean vs Soybean + 22:1	1	0.1	3	5.30
Among Tower[h]	1	0.9	6	4.90
Soybean[i] vs Tower[h]	1	8.6***	3	11.55**
Soybean[i] vs Olive[j]	1	2.1	3	1.65
Tower[h] vs Olive + 22:1	1	0.3	3	0.60

[a,b,c] See these footnotes Table III.

[d] One rat on this diet died during the 6th week.

[e] See footnote d, Table III.

[f,g] See footnotes f and g in Table III.

[h] Comparison among Tower, Tower + 0.5% 22:1 and Tower + 5.6% 22:1.

[i] Includes soybean and soybean + 5.7% 22:1.

[j] Does not include olive + 4.4% 22:1.

control oils.

The incidence and severity of heart lesions of rats fed in experiment II are given in Table IV. Again a definite but lower incidence and severity of heart lesions occurred in rats fed the control oils (soybean, olive) compared to rats fed Tower RSO or Tower RSO with added free erucic acid The addition of free 22:1 to soybean oil did not increase the incidence of lesions compared to when soybean oil alone was fed. On the other hand, the addition of free 22:1 to olive oil more than doubled the incidence of myocardial necrosis. Statistical analysis of these data revealed no significant differences in the incidence or severity of heart lesions between rats fed control oils or among rats fed rapeseed oils. The addition of free 22:1 to Tower oil or soybean oil did not significantly alter the incidence or severity of heart lesions. On the other hand, adding free 22:1 to olive oil significantly ($P<0.001$) increased the incidence and severity of heart necrosis.

Rats fed Tower oil developed a significantly higher incidence ($P<0.001$) and severity ($P<0.01$) of myocardial lesion than did rats fed soybean oil regardless of whether or not free 22:1 was added to either. On the other hand, soybean or soybean + 22:1 fed rats developed the same incidence and severity of myocardial necrosis as did rats fed olive oil. Similarly, rats fed olive oil with the addition of 22:1 developed the same incidence and severity of heart lesions as did rats fed Tower oil, with or without additional 22:1.

It has been postulated (4,9,10) that the cardiopathogenicity of rapeseed oil is due to the imbalanced fatty acid composition of the oil for the growing rat. It has been suggested that the high 18:1 of rapeseed oil in particular along with the presence of other long chain monoenes (20:1 and 22:1) and high 18:3 plays an important role in this postulated imbalance hypothesis (4,9,10). The results presented here support this hypothesis in that the addition of 22:1 to a control oil (olive), naturally high in 18:1 greatly increased both the incidence and severity of myocardial necrosis compared to when olive oil without 22:1 addition was fed.

The high content of 18:3 in rapeseed oils has also been suggested as the cause of cardiopathogenicity when that oil is fed to growing rats (6). The data presented here, however, do not support this suggestion in that adding 22:1 to a control oil (soybean), naturally high in 18:3, did not alter the incidence or severity of myocardial lesions. Neither did high 18:3 and the presence of high 20:1 and 5.6% 22:1 in an earlier study (4) – RPF diet, increase the incidence of heart lesions compared to that found in rats fed commercial lard.

There are many reports in the literature which indicate that the cardiovascular damage brought on by various dietary stress situations is not uncommon in growing rodents. In one particular study (18), more than 50% of the mice fed sugar, lard, and casein diets showed atrial myocardial necrosis with formation of mural thrombi, calcification, and fibrosis. In another report (19), 32% to 71% of the mice fed diets containing synthetic saturated triglycerides developed myocardial lesions (necrosis, fibrosis, calcification, and ceroid pigment deposits). Other workers (20) listed 13 possible dietary inclusions and/or alterations which can lead to myocardial necrosis and mineralization in rodents. More recently (15,16) this laboratory showed that the presence of excess oil in the diet of the young growing rat may also create a stress situation and thereby influence the incidence of myocardial necrosis and/or fibrosis.

In light of the above, the significance and meaning of these myocardial lesions should be put in perspective. It should be emphasized that this is not, and never has been, an "all or none" problem since a background incidence of myocardial necrosis considered by some to be "spontaneous" (1) and by others "normal" (2), occurs in male rats irrespective of diet (17). High rapeseed oil intake appears merely to exacerbate a problem which is present in the rat in any case

To conclude, from the data presented here, it would appear that the increased incidence of myocardial necrosis in male rats over background incidence, brought on by feeding rapeseed oils, is due to the imbalanced fatty acid composition of that oil in relation to the fatty acid requirements of the growing rat. It appears that this imbalance results from a combination of low saturates (4), high monoenes (18:1, 20:1 and 22:1), high 18:3, and a low 18:2 to 18:3 ratio, and not the mere presence of high 18:3 (6) or 22:1 (1). Differences in the requirement and/or metabolism of fatty acids may also explain the absence of myocardial lesions in chickens (21) and nonalbino (Chester Beatty) rats (16) fed rapeseed oils, and the lack of increased incidence in female rats (10,15), pigs (22,23), and monkeys (manuscript in preparation) when fed rapeseed oils compared to control oils.

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Ketone Bodies Serve as Important Precursors of Brain Lipids in the Developing Rat

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ABSTRACT

Ketone bodies are readily oxidized for energy by extrahepatic tissues. Since oxidation of ketone bodies produces acetyl coenzyme A (AcCoA), and hence could be an important source of immediate precursors for fatty acid synthesis, we investigated, in whole-brain homogenates of developing rats, the preferential utilization of [3-^{14}C]acetoacetate (AcAc), [3-^{14}C]β-hydroxybutyrate (β-OHB), and [U-^{14}C]glucose for production of CO_2 and lipids, including phospholipids, glycerides, cholesterol, and free fatty acids. Throughout the postnatal period, the rate of AcAc oxidation was 2-3 and 2-6 times the rate for β-OHB and glucose, respectively. The synthesis of lipids from AcAc was 7- to 11-fold higher than from glucose. The brain's capacity for lipid synthesis from β-OHB was similar to that from AcAc during the first 8 days of life; however, during the next 10 days, the synthesis of lipids from β-OHB decreased to 60% of AcAc-dependent synthesis. The high rate of lipid synthesis from ketone bodies was accompanied by increased activities of cytoplasmic acetoacetyl CoA synthetase and acetoacetyl CoA thiolase in the developing brain. During the entire postnatal development, the proportion of radioactivity claimed by lipids vs. CO_2 from [3-^{14}C]AcAc was 44-62% vs. 38-56%; from [3-^{14}C]β-OHB, 50-81% vs. 19-50%; and from [U-^{14}C]glucose, 14-43% vs. 57-86%. Phospholipids accounted for more than two-thirds of total lipids synthesized from either ketone bodies or glucose, while diglycerides plus cholesterol and free fatty acids accounted for most of the remainder. Addition of glucose to the incubation medium did not alter lipid production from AcAc throughout the suckling period, but moderately depressed energy production in the brain of 16- to 20-day-old rats. It is clear that in cell-free preparations from the brain of developing rats, ketone bodies are preferred over glucose as precursors for both energy and lipids, mainly phospholipids. These results suggest that ketone bodies are important for the growth and development of the brain.

INTRODUCTION

Hyperketonemia occurs during the early postnatal development in most mammalian species. In rats, levels of plasma β-hydroxybutyrate (β-OHB) and acetoacetate (AcAc) are low at birth but increase rapidly after the first postnatal feeding and remain high until weaning (1-4). The elevated ketone bodies (KB) can readily be oxidized to provide energy in extrahepatic tissues, particularly in the brain (5,6). Oxidation of KB in the brain is coupled to the tricarboxylic acid cycle (6), which is preceded by formation of acetyl CoA (AcCoA) (7). During the first 2 weeks of life, coincident with low levels of plasma glucose (1,3), activities of key glycolytic enzymes (e.g., hexokinase, phosphofructokinase, and pyruvate kinase) are low in the brain (8). Ketone bodies, by providing AcCoA, could therefore serve as important precursors for synthesis of brain lipids in the developing rat (9). Recently, Edmond (10) and Patel and Owen (11) showed that β-OHB and AcAc are incorporated into fatty acids and sterols in the brain of suckling rats. Since brain lipids contain predominantly phospholipids, sphingolipids, and cholesterol during the early postnatal development (12), we investigated the rates of utilization of KB and glucose for synthesis of complex lipids in the brain. The results show that AcAc and β-OHB are preferred over glucose as substrates for phospholipids and other lipids.

MATERIALS AND METHODS

Animals

Female Sprague-Dawley rats were bred and kept in individual cages as previously described (13). They were fed Purina rat chow and water ad libitum. Postnatal rats of 1- to 22-days-old were suckled by their dams until the experiments were begun, whereas newborn rats obtained by caesarean section were used immediately after birth and before postnatal feeding. Adult rats (200-250 g) maintained on Purina rat

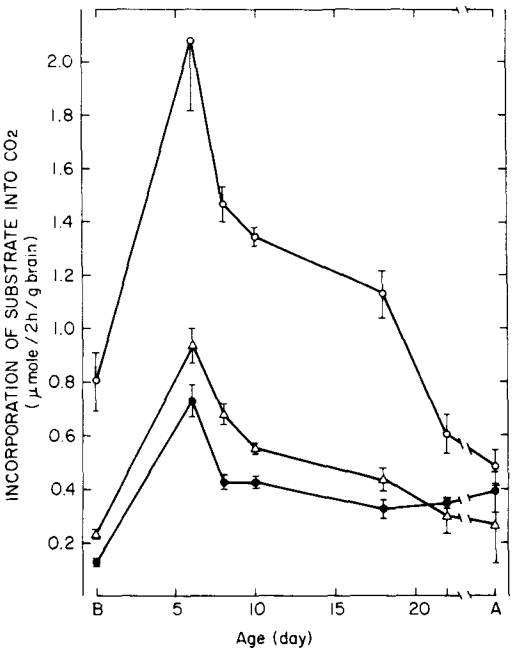


FIG. 1. Oxidation of ketone bodies and glucose in the brains of developing rats. The brain homogenates were incubated for 2 hr in a final 2 ml of Ca^{2+}-free Krebs-Riger bicarbonate buffer containing 20 μmoles and 0.5 μCi of ^{14}C-labeled substrates. The rate of $^{14}CO_2$ production from [3-^{14}C]acetoacetate (○——○), [3-^{14}C]β-hydroxybutyrate (△——△), or [U-^{14}C]glucose (●——●) was expressed as μmoles of substrate incorporated/2 hr per g of brain (wet wt). Values represent means ± SEM for four to six samples. Each sample consisted of brains from one to four rats. B denotes newborn rats at birth, and A, adult rats fed ad libitum.

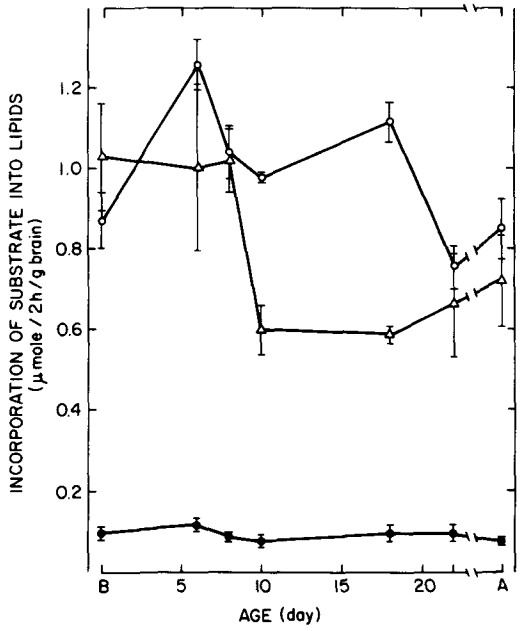


FIG. 2. Synthesis of lipids from ketone bodies and glucose in the brains of developing rats. The brain homogenates were incubated for 2 hr in a final 2 ml of Ca^{2+}-free Krebs-Ringer bicarbonate buffer containing 20 μmoles and 0.5 μCi of [3-^{14}C]acetonacetate (○——○), [3-^{14}C]β-hydroxybutyrate (△——△), or [U-^{14}C]- glucose (●——●). The rate of synthesis of lipids expressed as μmoles of substrate incorporated/2 hr per g of brain (wet wt) represents total synthesis of phospholipid, monoglyceride, diglyceride, triglyceride, free fatty acid, cholesterol, and cholesteryl ester. Values represent means ± SEM for four to six samples. Each sample consisted of brains from one to four rats. B denotes newborn rats at birth, and A, adult rats fed ad libitum.

chow were also included in this study for comparison.

Metabolic Studies

Immediately after the rats were decapitated, their brains were excised and placed in ice-cold saline (0.9% NaCl). Whole brains (1.5-2.0 g pooled from 1 to 4 rats) were homogenized in 3 volumes of Ca^{2+}-free Krebs-Ringer bicarbonate buffer (pH 7.4) to obtain a crude homogenate, 25% (w/v). After centrifugation at 400 x g for 5 min at 4 C, the resulting homogenate supernatant was incubated for matabolic study. The incubation system in a total of 2 ml consisted of Ca^{2+}-free Krebs-Ringer bicarbonate buffer (pH 7.4), 1.2 ml; homogenate supernatant, 0.8 ml; bovine serum albumin, 30 mg; substrate ([3-^{14}C]acetoacetate, [3-^{14}C]β-hydroxybutyrate, or [U-^{14}C] glucose), 20 μmoles (0.5 μCi). At the end of 2 hr of incubation in a metabolic shaker (90 strokes/min) at 37 C, the reaction was stopped by adding 0.5 ml of 2 N H_2SO_4. It was observed that both oxidative and synthetic activities increased linearly with time of incubation up to 2 hr. The CO_2 produced from [^{14}C]substrates was trapped in filter paper and determined in the same manner as reported earlier (13). After CO_2 collection, a 1-ml aliquot of the incubation mixture was used for lipid extraction (14). Lipids of different classes were separated by thin layer chromatography (13). Radioactivity present in separate lipid classes and in CO_2 was determined with a liquid scintillation counter (Mark III, Searle, Inc.). The rate of synthesis was expressed as μmoles of substrate incorporated into product/2 hr per g of wet brain.

Enzyme Assays

Cytoplasmic fractions (105,000 x g supernatant) of brain prepared in a homogenizing medium containing 10 mM tris buffer (pH 7.4), 0.25 M sucrose, and 1 mM 2-mercaptoethanol

TABLE I

Percent Distribution of [3-^{14}C]acetoacetate in Lipids Synthesized in the Brains of Developing Rats

Age (day)	Lipid classes[a]				
	PL + MG	DG + C	FFA	TG	CE
At birth	72.6 ± 2.5[b]	15.3 ± 2.0	9.5 ± 0.9	1.4 ± 0.1	1.3 ± 0.3
6	67.9 ± 2.4	13.9 ± 1.0	14.7 ± 1.9	1.1 ± 0.5	2.4 ± 0.8
10	73.3 ± 1.4	10.2 ± 0.9	13.1 ± 0.6	1.6 ± 0.2	2.0 ± 0.1
16	70.4 ± 1.8	12.0 ± 0.5	15.9 ± 1.9	0.8 ± 0.1	1.0 ± 0.2
18	77.4 ± 2.6	13.4 ± 1.7	5.5 ± 1.2	3.2 ± 0.5	0.5 ± 0.2
22	75.7 ± 2.3	11.2 ± 0.5	8.6 ± 1.2	3.9 ± 0.7	0.6 ± 0.3
Adult	78.7 ± 2.1	12.2 ± 0.9	6.6 ± 0.9	2.0 ± 0.3	0.5 ± 0.2

[a]Lipid classes including phospholipid (PL), monoglyceride (MG), diglyceride (DG), cholesterol (C), free fatty acid (FFA), triglyceride (TG), and cholesteryl ester (CE) were determined by thin layer chromatography. The distribution of acetoacetate radioactivity in individual lipids is expressed as the percentage of activity incorporated into total lipids.

[b]Values are means ± SEM for four to six samples. Each sample consisted of brains from one to four rats.

TABLE II

Percent Distribution of [3-^{14}C]β-hydroxybutyrate in Lipids Synthesized in the Brains of Developing Rats

Age (day)	Lipid classes[a]				
	PL + MG	DG + C	FFA	TG	CE
At birth	90.8 ± 0.3[b]	6.4 ± 0.4	2.2 ± 0.1	0.4 ± 0.1	0.2 ± 0.1
6	88.8 ± 3.4	7.2 ± 1.6	3.6 ± 0.5	0.2 ± 0.02	0.4 ± 0.2
18	92.3 ± 0.7	5.3 ± 0.6	2.1 ± 0.2	0.2 ± 0.1	0.2 ± 0.1
22	85.8 ± 1.0	5.4 ± 0.8	6.3 ± 0.9	2.2 ± 0.9	0.2 ± 0.1
Adult	80.4 ± 4.6	13.0 ± 3.2	5.0 ± 1.2	1.2 ± 0.5	0.4 ± 0.1

[a]Lipid classes including phospholipid (PL), monoglyceride (MG), diglyceride (DG), cholesterol (C), free fatty acid (FFA), triglyceride (TG), and cholesteryl ester (CE) were determined by thin layer chromatography. The distribution of β-hydroxybutyrate radioactivity in individual lipids is expressed as the percentage of activity incorporated into total lipids.

[b]Values are means ± SEM for four to six samples. Each sample consisted of brains from one to four rats.

were used to assay ketone utilizing enzymes.

As determined from the activity of glutamic dehydrogenase (15), there was no appreciable contamination with mitochondrial matrix enzymes in the prepared cytoplasm. The activity of the dehydrogenase in cytoplasm was less than 0.1% of that observed in mitochondria. The activity of acetoacetyl CoA synthetase was determined by coupled reactions involving malate dehydrogenase, citrate synthase, and acetoacetyl CoA thiolase (16). The assay system contained, in 1 ml, tris buffer (pH 8.5), 75 μmoles; $MgCl_2$, 5 μmoles; L-malate, 2 μmoles; ATP, 1 μmole; CoA, 0.025 μmole; NAD^+, 9 μmoles; acetoacetate, 2.5 μmoles; malate dehydrogenase, 4 units; citrate synthase, 0.18 unit. An increase in absorbance due to the formation of NADH was measured with a Gilford spectrophotometer (Model 250). The activity of acetoacetyl CoA thiolase (EC 2.3.1.9) was determined by the method of Williamson et al. (17).

Chemicals

DL-[3-^{14}C]β-hydroxybutyrate, ethyl[3-^{14}C]acetoacetate, and [U-^{14}C]glucose were products of New England Nuclear, Boston, MA. [3-^{14}C] acetoacetate was synthesized from ethyl[3-^{14}C]acetoacetate according to the method of Krebs and Eggleston (18). Malate dehydrogenase and citrate synthase were purchased from Boehringer Mannheim Biochemicals, Indianapolis, IN. Coenzyme A and acetoacetyl CoA were obtained from P.L. Biochemicals, Inc., Milwaukee, WI. Bovine serum albumin (Fraction V), ATP, and other chemicals were from Sigma Chemicals Co., St. Louis, MO.

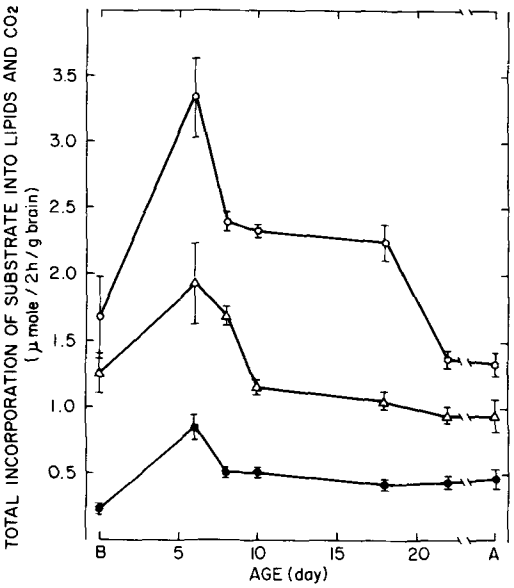


FIG. 3. Total utilization of ketone bodies and glucose for production of lipids and CO_2 in the brains of developing rats. The data are derived from the experiments depicted in Figures 1 and 2. The rate of total production of lipids and CO_2 from [3-^{14}C]acetoacetate (o———o), [3-^{14}C] β-hydroxybutyrate (Δ———Δ), or [U-^{14}C]glucose (•———•) is expressed as μmoles of substrate incorporated/2 hr per g of brain (wet wt). Values represent means ± SEM for four to six samples. Each sample consisted of brains from one to four rats. B denotes newborn rats at birth, and A, adult rats fed ad libitum.

RESULTS

In preliminary studies, maximal rates of CO_2 and lipid production were obtained with 10 mM each of AcAc, β-OHB, or glucose during 2 hr of incubation of brain homogenates. Subsequent experiments were carried out under these conditions.

At birth, the rate of AcAc oxidation to CO_2 was 0.81 ± 0.12 μmole/2 hr per g of brain, or 68% higher than that of the adult rat brain. On the 6th day of life, the rate increased to 2.6-fold of the initial rate but gradually declined thereafter, reaching the adult level at weaning (Fig. 1). When β-OHB was used as the substrate, a developmental pattern similar to AcAc oxidation was observed; however, the rates of β-OHB oxidation were only one-third to one-half of AcAc oxidation throughout the entire postnatal period. The developmental changes in glucose oxidation also followed that of CO_2 production from AcAc, and the rates of oxidation were lower than that of β-OHB and AcAc. In adult fed rats, the production of CO_2 from these three substrates was the same.

We then measured the incorporation of KB and glucose into lipids, including phospholipids (PL), mono-, di-, and triglycerides (MG, DG, TG), cholesterol (C), cholesteryl ester (CE), and free fatty acids (FFA). In newborn rats, the brain's capacity for synthesis of lipids from AcAc was comparable to that in adults (Fig. 2). Incorporation of AcAc into lipids increased during the suckling period but dropped to the adult rate at weaning. During the first 8 days of postnatal development, synthesis of brain lipids from β-OHB was as high as that from AcAc. Between days 10 and 18, however, the rate of synthesis from β-OHB decreased to about 60% of that from AcAc. The synthesis of lipids from glucose equaled only 10% of that from AcAc observed at birth. The capacity to synthesize lipids from glucose remained low throughout postnatal development and adulthood.

Using thin layer chromatography, we separated different lipids, i.e., phospholipids, glyc-

TABLE III

Percent Distribution of [U-^{14}C]glucose in Lipids Synthesized in the Brains of Developing Rats

Age (day)	Lipid classes[a]				
	PL + MG	DG + C	FFA	TG	CE
At birth	68.0 ± 3.2[b]	9.7 ± 1.0	11.5 ± 2.0	4.0 ± 0.6	6.7 ± 1.3
6	70.8 ± 3.5	8.7 ± 1.4	13.7 ± 1.0	3.2 ± 0.2	3.6 ± 1.4
12	66.8 ± 2.0	12.1 ± 1.1	14.5 ± 1.9	2.4 ± 0.4	4.4 ± 0.9
18	63.1 ± 3.4	11.4 ± 1.1	18.1 ± 2.7	4.0 ± 0.9	3.5 ± 1.6
Adult	71.0 ± 2.7	5.4 ± 2.3	15.7 ± 2.6	5.4 ± 2.2	2.5 ± 0.3

[a]Lipid classes including phospholipid (PL), monoglyceride (MG), diglyceride (DG), cholesterol (C), free fatty acid (FFA), triglyceride (TG), and cholesteryl ester (CE) were determined by thin layer chromatography. Distribution of glucose radioactivity in individual lipids is expressed as the percentage of activity incorporated into total lipids.

[b]Values are means ± SEM for four to six samples. Each sample consisted of brains from one to four rats.

TABLE IV

Partitioning of Acetoacetate, β-hydroxybutyrate, and Glucose into Lipids and CO_2 in the Brains of Developing Rats

	Percent incorporation of:[a]					
	[3-^{14}C]acetoacetate		[3-^{14}C]β-hydroxybutyrate		[U-^{14}C]glucose	
Age (day)	Lipids	CO_2	Lipids	CO_2	Lipids	CO_2
At birth	53.6 ± 1.7[b]	46.4	81.4 ± 1.4	18.6	43.1 ± 2.9	56.9
6	37.8 ± 1.9	62.2	50.2 ± 4.1	49.8	13.6 ± 0.9	86.4
8	38.6 ± 1.4	61.4	60.1 ± 0.9	39.9	17.2 ± 0.6	82.8
10	42.2 ± 0.6	57.8	52.3 ± 1.6	47.7	15.7 ± 1.1	84.3
18	49.9 ± 1.2	50.1	58.0 ± 2.2	42.0	23.2 ± 1.5	76.8
22	55.6 ± 4.0	44.4	69.0 ± 2.7	31.0	22.4 ± 3.9	77.6
Adult	63.7 ± 2.5	36.3	73.1 ± 5.3	26.8	15.8 ± 3.7	84.2

[a]Percent incorporation into each fraction was determined on the basis of total radioactivity of the substrates recovered in CO_2 and lipids, including phospholipid, glyceride, cholesterol, cholesteryl ester, and free fatty acids.

[b]Values are means ± SEM for four to six samples. Each sample consisted of brains from one to four rats. SEM for CO_2 production are the same as that for lipids.

TABLE V

Effect of Glucose on Incorporation of [3-^{14}C]acetoacetate into Lipids and CO_2 in the Brains of Developing Rats[a]

	μmoles incorporated/2 hr per g wet wt			
	Lipids		CO_2	
Age (day)	- Glucose	+ Glucose	- Glucose	+ Glucose
At birth	0.87 ± 0.07[b]	0.82 ± 0.13	0.80 ± 0.12	0.67 ± 0.03
6	1.25 ± 0.06	1.24 ± 0.07	2.08 ± 0.28	2.12 ± 0.21
8	0.92 ± 0.07	0.98 ± 0.04	1.47 ± 0.06	1.34 ± 0.06
16	1.04 ± 0.06	1.17 ± 0.09	1.33 ± 0.03	1.13 ± 0.07*[c]
18	1.12 ± 0.05	1.14 ± 0.04	1.15 ± 0.05	0.93 ± 0.07*
20	0.69 ± 0.02	0.73 ± 0.10	1.25 ± 0.08	0.91 ± 0.08*

[a]The brain homogenates were incubated for 2 hr in a final 2 ml of Ca^{2+}-free Krebs-Ringer bicarbonate buffer containing 20 μmoles and 0.5 μCi of [3-^{14}C]acetoacetate with or without 20 μmoles of nonlabeled glucose.

[b]Values are means ± SEM for four samples. Each sample consisted of one to four rats.

[c]*Indicates statistically significant difference between controls and glucose-treated groups at $p < 0.05$.

erides, cholesterol, cholesteryl esters, and free fatty acids, synthesized from ^{14}C-labeled substrates. The distribution of the radioactivity from [3-^{14}C]AcAc in various lipids is shown in Table I. Of the total lipids produced in suckling rats, PL + MG accounted for 68-77%, DG + C for 10-15%, and FFA for 6-16%. Small amounts of TG and CE were synthesized from [3-^{14}C]AcAc. The radioactivity distribution pattern for adult rats was similar to that for developing rats. When [3-^{14}C]β-OHB was used as substrate, the radioactivity incorporated into PL + MG accounted for more than 86% in newborn and suckling rats (Table II). The high rate of synthesis of this lipid fraction resulted in less incorporation of [3-^{14}C]β-OHB into DG + C and FFA. Low amounts of radioactivity were also found in TG and CE. In adult brain, the percent distribution of [3-^{14}C]β-OHB in the various lipid fractions was similar to that of [3-^{14}C]AcAc (Table I). The distribution of [U-^{14}C]glucose in lipids resembled the pattern for [3-^{14}C]AcAc (Tables I and III) in both developing and adult rats. The bulk of lipids synthesized from glucose were PL + MG (67-71%), FFA (12-18%), and DG + C (5 to 12%). Using a solvent system consisting of chloroform, acetone, and glacial acetic acid (88:12:0.05, v/v/v), we separated PL from MG in some experiments. The results showed that PL represented more than 98% of the PL + MG fraction derived from ^{14}C-labeled substrates.

The total incorporation of substrates into lipids and CO_2 was determined (Fig. 3). At birth, AcAc and β-OHB are utilized equally for synthesis of lipids and CO_2. After birth and throughout the suckling period, twice as much AcAc as β-OHB was utilized. Further, while

TABLE VI

Specific Acitivities of Cytoplasmic Acetoacetyl CoA Synthetase and Acetoacetyl CoA Thiolase in the Brains of the Developing Rat

Age (day)	nmoles AcCoA removed or formed/min per g wet wt	
	AcAc CoA synthetase	AcAc CoA thiolase
5	12.7 ± 1.8[a]	1432 ± 71
9	16.4 ± 1.6	1404 ± 131
13	18.4 ± 0.3	2396 ± 230
17	15.2 ± 1.1	1661 ± 47
21	18.8 ± 1.0	1167 ± 59
Adult	6.3 ± 0.5	1284 ± 153

[a]Values represent means ± SEM for four to six samples. Abbreviations: AcAc CoA, acetoacetyl CoA; AcCoA, acetyl CoA.

β-OHB utilization decreased to the adult capacity within 10 days of life, AcAc incorporation remained at a rate higher than the adult until weaning. The total utilization of glucose in developing rats was the lowest among the substrates studied. In contrast to AcAc and β-OHB, glucose was utilized less at birth than during adulthood. Six days after birth, however, glucose utilization exceeded the adult leve, returning to it 8 days after birth.

To determine the preference of brain tissue for substrate utilization via oxidative and synthetic processes, we estimated the proportions of KB and glucose metabolized for energy and lipid production. Of the total AcAc utilized over the entire postnatal period, 38 to 56% was converted to lipids and 44 to 62% to CO_2 (Table IV). The percent incorporation of β-OHB into lipids (50 to 81%) was higher than that of AcAc, consistent with a decreased incorporation (19 to 50%) of β-OHB into CO_2. On the other hand, 57 to 86% of glucose was metabolized to CO_2 and only 14-43% was synthesized into lipids. In adult rats, the relative incorporation of AcAc into lipids vs. Co_2 was 64 vs. 36%; β-OHB, 73 vs. 27%; and glucose, 16 vs. 84%. These results indicate that glucose is preferentially oxidized for energy, whereas AcAc and β-OHB are utilized about equally for energy and lipid synthesis. This led us to determine the effect of glucose on production of lipids and CO_2 from AcAc. In these experiments, [3-^{14}C] AcAc (10 mM) was incubated with brain homogenates in the presence or absence of nonlabeled glucose (10 mM). Throughout the entire postnatal development, the incorporation of [3-^{14}C] AcAc into lipids was not altered by glucose (Table V). The addition of glucose, however, depressed CO_2 production from [3-^{14}C] AcAc in 16-, 18-, and 20-day-old rats but had no effect on younger pups.

In an attempt to delineate the possible mechanisms of rapid conversion of KB to lipids, we measured activities of AcAc CoA synthetase and AcAc CoA thiolase in brain cytoplasm. These enzymes are of importance in providing cytoplasmic AcCoA (16). In suckling rats, the activities of AcAc CoA synthetase were two to three fold of the adult brain (Table VI). The activity of AcAc CoA thiolase in suckling rats was at least as high as that of adult animals. A similar developmental pattern of the thiolase activity has been reported previously (3). Since the activities of the thiolase were 60- to 130-times higher than the synthetase activity in developing rats, the rapid synthesis of lipids from KB is likely associated with the elevated activity of AcAc CoA synthetase.

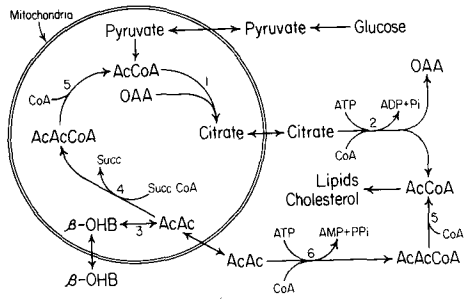
DISCUSSION

Earlier studies by others have shown that AcAc and β-OHB are readily oxidized by cerebral cortex slices in vitro (5) and by the whole brain in vivo (6). More recent investigations have indicated that KB are also utilized for fatty acid and sterol synthesis by the brain (10,11). Using cell-free preparations, we have demonstrated that capacities for the brain of developing rats to synthesize complex lipids and energy from KB are higher than that from glucose (Figs. 1 and 2). For example, the rate of AcAc oxidation, expressed as μmoles of substrate incorporated/2 hr per g brain (wet wt), was approximately 2 to 6 times the rate for glucose. The rate of incorporation of AcAc into lipids was 8 to 12 times that of glucose. The total incorporation of glucose into CO_2 plus complex lipids amounted to only 13 to 32% of AcAc incorporation and 18 to 43% of β-OHB incorporation. Further, there were distinct differences in the partitioning of substrates into energy and lipids. For every portion of glucose incorporation into lipids during the suckling period, three to six portions are metab-

olized to CO_2, suggesting that glucose is preferentially utilized for energy. By contrast, during the same period of development, the amount of AcAc oxidized to CO_2 was only about 1 to 1.5 times the amount metabolized to lipids. When β-OHB was the substrate, the ratios of CO_2 vs. lipid incorporation, ranging from 0.4 to 0.9, were even lower than that of AcAc. It is important to note that in intact brain, utilization of KB depends not solely on enzymatic machinery but also on concentrations and transport of the substrates from peripheral circulation (2). Thus, under conditions in which plasma levels of KB increase while glucose levels remain low (3,4) during the early postnatal period, KB become important precursors not only for energy but for lipid synthesis in the brain.

In rats, phospholipids, cholesterol, and sphingolipids comprise most of the brain lipids (12). The brain grows rapidly after birth and continues to increase in size and maturity throughout the suckling period. During this rapid growth phase, the brain contains increasing amounts of the major lipid components. These observations, coupled with the high incorporation of AcAc and β-OHB into phospholipid and cholesterol plus diglyceride, suggest that the supply of KB is essential for the growth and development of the brain. The importance of KB for the developing rat is further emphasized by the observation that glucose could not displace AcAc for lipid synthesis.

Although the precise mechanism is not known, the accelerated rates of AcAc and β-OHB oxidation observed in the brain of suckling rats are likely associated with increased activities of ketone-utilizing enzymes – i.e., β-hydroxybutyrate dehydrogenase (2,9), 3-oxoacid CoA transferase (2,3), and acetoacetyl CoA thiolase (3) – and increase availability of plasma KB (2-4). The basis for the higher rates of lipid synthesis from AcAc and β-OHB than from glucose is not clear. As proposed in Scheme 1, lipogenesis depends upon the availability of AcCoA in cytoplasm (19). Since oxidation of glucose generates AcCoA in mitochondria, the incorporation of glucose into lipids could be limited by ATP citrate lyase, which is required for the transport of mitochondrial AcCoA to cytoplasm (19,20). Oxidation of AcAc via 3-oxoacid CoA transferase and AcAc CoA thiolase reactions also results in production of mitochondrial AcCoA, which is then subjected to the regulation of citrate synthease and ATP citrate lyase as in glucose metabolism (7,16). Alternatively, AcAc can be activated by cytoplasmic AcAc CoA synthetase to form AcAc CoA, which is then deacetylated



SCHEME 1. Proposed scheme for the synthesis of cytoplasmic acetyl CoA from ketone bodies and glucose. Key to enzymes: 1. citrate synthase; 2. ATP-citrate lyase; 3. β-hydroxybutyrate dehydrogenase; 4. 3-oxoacid CoA transferase; 5. acetoacetyl CoA thiolase; 6. acetoacetyl CoA synthetase. Abbreviations: AcAc, acetoacetate; AcCoA, acetyl CoA; AcAcCoA, acetoacetyl CoA; CoA, coenzyme A; β-OHB, β-hydroxybutyrate; OAA, oxaloacetate; Succ, succinate; Succ CoA, succinyl CoA.

by cytoplasmic AcAc CoA thiolase to AcCoA (16,21). In the present study, we observed high activities of cytoplasmic AcAc CoA synthetase and AcAc CoA thiolase in the brains of developing rats. It is therefore likely that the rapid incorporation of KB into lipids is in part attributable to an increased production of AcCoA through this cytoplasmic pathway.

Throughout the suckling period, the rates of β-OHB oxidation to CO_2 were lower than that of AcAc. The incorporation of β-OHB into lipids was also lower than that of AcAc during the second half of suckling stage, when the activity of β-OHB dehydrogenase had increased substantially (2,9). Since AcAc has been shown to be more permeable than β-OHB (22), the metabolism of the latter might be limited by its uptake in the brain.

The present data, derived from studies with whole-brain homogenates, disclosed only general developmental changes of lipid metabolism in the brain. Since all parts of the brain do not necessarily consist of the same lipid components, or develop at the same rate (12,23), the physiological function of KB in a given region of the brain remains to be established.

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Autoxidation of Fatty Acid Monolayers Adsorbed on Silica Gel: I. Nature of Adsorption Sites

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ABSTRACT

An unsaturated fatty acid monolayer deposited on a silica gel surface has been chosen as a model for studying non-enzymatic autoxidation of membrane lipids. Studies to determine the suitability of this system as a model for biomembranes were conducted to define the nature of the monolayers, particularly with respect to the factors determining the concentration of the fatty acid molecule on unit area of the surface. The results from adsorption isotherm, high temperature dehydroxylation, and infrared spectra studies show that adsorption of a monomolecular layer of fatty acids occurs and that the number of molecules absorbed corresponds to the number of isolated, non-hydrogen-bonded silanol groups. It is presumed the binding is by hydrogen bonding of the carboxyl groups to silanol groups. The packing density of the fatty acid molecules is 1.25 molecule/100 Å^2 which is similar to the density of the isolated silanol sites on the surface.

INTRODUCTION

Since the recognition of the differences in oxygen-related reactions in bulk phase and membrane-like ordered arrangement, numerous studies on membrane peroxidation have been carried out largely by using tissue homogenates and isolated membrane fragments (1-4). The results from such approaches, however, are sometimes contradictory and inconclusive because of the complexity of the systems involved. For this reason, many investigators have turned their attention to simpler, chemically well-defined monolayer and bilayer model systems for studying non-enzymatic membrane peroxidation.

There are two known types of monolayer preparations; those deposited on a solid surface and those spread on an air-water interface. Both types of lipid monolayers have been utilized by investigators as models for autoxidation studies. We chose to use the deposited monolayers to gain further knowledge in membrane autoxidation for the following reasons: (a) the preparation is relatively simple and highly reproducible, (b) the particular system of interest, unsaturated fatty acids adsorbed on silica gel, has been widely utilized in this laboratory in connection with other studies, and (c) in air-water systems, unsaturated fatty acids spread on water are known to dissolve to a measurable extent in the water (5).

There have been several autoxidation studies using monolayers adsorbed on inert solid support (6-10). Honn and co-workers (6) used silica gel as adsorbent to measure the rate of autoxidation of soybean oil, and Porter et al. (7) used pure linoleic acid monolayers adsorbed on silica gel to study the effects of prooxidants (metals and acid synergists) and an antioxidant (α-tocopherol) on the rate of lipid autoxidation. In these studies, the question of the exact nature of the monolayer system with respect to the type of adsorption sites and the orientation of lipid molecules was ignored entirely except for a brief statement by Porter (7) that the possibility of multilayer formation was eliminated by their adsorption isotherm.

Our feeling of urgency in experimentally defining this system stems from a desire to settle the discrepancy existing between our observation on the area occupied by a linoleic acid molecule (about 80 Å^2) obtained at the maximum adsorption and the area/molecule of saturated fatty acids (20-23 Å^2) from monolayers spread on an air-water interface under lateral compression (11,12).

Amorphous silica gel surfaces have been characterized to some extent in recent years, in terms of the dehydration behavior and the concentration and types of silanol groups present (13,14). Using partly the available knowledge of the silica surface itself, we sought in the present work to determine the governing features of the monolayer adsorption process in fatty acid-silica gel systems and to use the results to obtain a realistic view of the similarities and dissimilarities of this model to biomembranes. This introductory paper is to be followed by other parts of the major study of monolayer autoxidation. In the second study, attention is primarily focussed on the rates and products of autoxidation in such monolayers, which are shown to be different from bulk phase autoxidation. In succeeding studies, the effects on the autoxidation rate of incorporating prominent

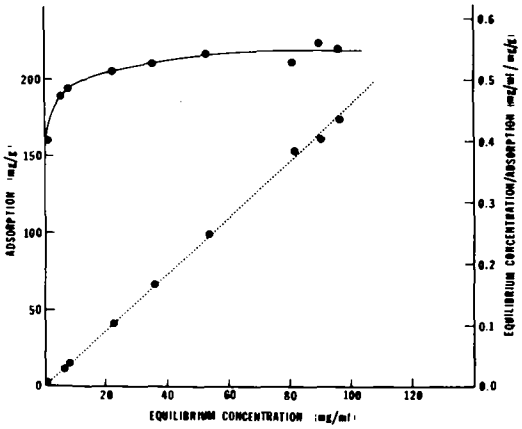

FIG. 1. Conventional (—●—) and Langmuir (---●---) plots of adsorption of linoleic acid on Silica Gel G at 24 C as function of equilibrium concentration of hexane solution.

membrane constituents and antioxidants into linoleic acid monolayers will be examined.

EXPERIMENTAL PROCEDURES

Materials and Methods

Linoleic, linolelaidic, and oleic acids purchased from Applied Science Laboratories, Inc. (State College, PA) are 99+% pure and were used directly. The purity of these compounds was routinely checked by gas liquid chromatography (GLC) and thin layer chromatography (TLC) to insure purity specified by the manufacturer. 12-Bromo-*trans*-9-octadecenoic acid was prepared by brominating 12-hydroxy-*trans*-9-octadecenoic acid (ricinelaidic acid) with carbon tetrabromide and triphenylphosphine (R.A. Stein, unpublished results). The product was isolated by silicic acid column chromatography and had a purity of at least 96%.

Silica Gel G and Silica Gel H were both supplied by E. Merck AG, Darmstadt, Germany. Both materials have a median grain size of 10-40 μ and contain 0.02% iron. Silica Gel G contains 13% calcium sulfate as binder and Silica Gel H contains no binder. By BET method (gas adsorption at cryogenic temperature), using nitrogen gas as adsorbate, Silica Gel G gives a specific surface area of 378 m^2/g and pore volume, 0.70 ml/g (determined by Pacific Sorption Service, Chico, CA). With these two values, average pore diameter was calculated to be 74 Å by the equation, average pre diameter = 4 x pore volume/specific surface area (15).

Determination of Adsorption Isotherm

Various amounts (ranging from 0.0840 g to 0.9204 g) of linoleic acid were dissolved in 7 ml of hexane (Phillips Petroleum Company, pure grade). Exactly 1 ml of this solution was withdrawn, and the concentration was determined gravimetrically after evaporation of solvent. To a 0.5 g portion of Silica Gel G, 5 ml of the above solution was added, and the contents were stirred for 45 min at 24 C in a tightly stoppered flask. Upon completion of the equilibration, 1 ml of the solution was withdrawn, and the amount of residue was determined as above. From the initial and final concentration, the amount of linoleic acid adsorbed per g of silica gel was calculated.

Dehydration of Silica Gel

For removing physically adsorbed water and hydrogen-bonded silanols from Silica Gel G or Silica Gel H, bulk silica gel was heated in a small round-bottomed flask connected to a vacuum line (0.1 mm Hg). The temperature was monitored by a pyrometer attached to thermocouple leads of the heating mantle. The weight losses at various stages of dehydration (or dehydroxylation) in a typical run are summarized below: starting with 4.0149 g of Silica Gel G, after heating at 200 C for 10 min, loss was 0.0670 g; 400 C for 10 min, a further 0.0228 g; 450 C for 1.5 hr, a further 0.0252 g.

For infrared measurements, silica gel samples (6 mg) were pressed in a stainless steel die at about 70 kg/cm^2 for a few seconds to form discs, 1.2 cm in diameter. The discs were heated for a desired length of time at various temperatures in vacuo or in a muffle oven. After heating, the discs were mounted immediately in a holder and positioned in the spectrometer. Both Baird Associates Model B and Infracord Model 137 (Perkin-Elmer Corporation) instruments were used for obtaining the infrared spectra.

RESULTS AND DISCUSSION

In order for our system to be valid as a membrane model, it is important to first establish the monolayer nature of the adsorbed fatty acid. The adsorption isotherm of linoleic acid at the n-hexane/silica gel interface obtained for this purpose is shown in Figure 1. It was found that the adsorption conforms to a Langmuir isotherm, showing a definite region of "limiting" adsorption characteristic of monolayer, exclusive of multilayer formation. The general shape of the curve resembles those obtained for linoleic acid at a petroleum ether/silica interface by Porter et al. (7). The physical characteristics of silica gel used by Porter et al. are similar to ours, the only difference being the specific surface areas measured by BET nitrogen

adsorption; thus, at the maximum adsorption, theirs gives 260 mg of linoleic acid/g of silica for an area of 450 m^2/g and ours, 220 mg of linoleic acid for 378 m^2/g, the value for area/molecule being about 80 $Å^2$ /molecule in both cases. Unlike Porter's, deviation of our individual determinations from the final curve determined by the method of least square (at the region of maximum adsorption) was found to be generally very small, thus eliminating any uncertainty associated with the shape of the curve. Porter and co-workers (7) reported briefly on the adsorption isotherm for the linoleic acid-silica gel system they used and apparently did not pursue any further investigation as to the nature of fatty acid binding sites.

The area/molecule value of 80 $Å^2$ for linoleic acid calculated from the adsorption isotherm is far from the reported values for cross-sectional area of fatty acids. For example, for stearic acid in an air-water interface system, the limiting area/molecule is 20.4-23.8 $Å^2$/molecule depending on the substrate pH (12,16). Oleic acid shows a slightly greater area/molecule, 26-27 $Å^2$ under the same condition (17) and an approximation of 30 $Å^2$ may be appropriate for linoleic acid. Measurements of a Stuart-Briegleb ball model of the stearic acid molecule in an extended linear configuration yield cross-section areas, 18.5 $Å^2$ and 21.3 $Å^2$, depending on the angle of rotation of the carboxyl group (18). A considerable departure from these values in our system clearly suggests that the linoleic acid monolayers are not closely packed arrays of fatty acid molecules oriented vertically to the surface as in the case of the air-water interface. Earlier research (19) showed that stearic acid required 65 $Å^2$/molecule when absorbed on dry silica gel, and it was recognized that this area was much larger than that required by consideration of the cross-sectional area only. Nonetheless, more recent work (20,21) postulated the adsorption of fatty acid on silica surface as a close-packing of molecules with hydrocarbon chains oriented parallel to the surface. Our results indicate (a) the area/molecule obtained at the maximum adsorption is far smaller than that expected with the molecule lying flat on the surface [about 143 $Å^2$ for linoleic acid (20)], (b) as shown in Table I, the number of moles adsorbed by the unit area of silica is insensitive to adsorbates with differences in length of hydrocarbon chain, number and isomerism of double bonds, and substitution in the hydrocarbon chain. These two lines of evidence prompt us to postulate that the carboxyl groups are held (probably by hydrogen bonds) to the specific adsorption sites situated farther apart than the cross-sectional area of fatty acid, thus preventing a closer packing of fatty acid molecules.

TABLE I

Amounts of Various Fatty Acids Adsorbed from Hexane per Gram of Silica Gel

Fatty acid	Millimoles adsorbed per gram of silica[a]
Linoleic	0.687 ± 0.005[b]
Palmitic	0.690
Linolelaidic	0.691
Oleic	0.680
12-Bromo-*trans*--9-octadecenoic	0.683

[a]At the equilibrium concentration of 10 mg/ml.
[b]Mean deviation.

TABLE II

Effect of Heat Treatment on the Loss of Surface Silanol Groups and Adsorption of Linoleic Acid

Silica gel	Heat treatment	Number of OH lost per 100 $Å^2$[d]	% of linoleic acid monolayer coverage
G	130 C/overnight	---	100.0[a]
	400 C/10 min[c]	2.35	97.2
	450 C/1.5 hr[c]	3.65	86.1
H	130 C/overnight	---	100.0[b]
	290 C/1.0 hr[c]	3.36	88.9

[a]The amount of 220 mg of linoleic acid/g of silica as seen in the adsorption isotherm (Fig. 1) was taken as 100.0%.
[b]The amount of 234 mg of linoleic acid/g of silica gel was taken as 100.0%.
[c]Carried out in 0.1 mm vacuum.
[d]The weight losses used for this calculation exclude the loss due to physically adsorbed water.

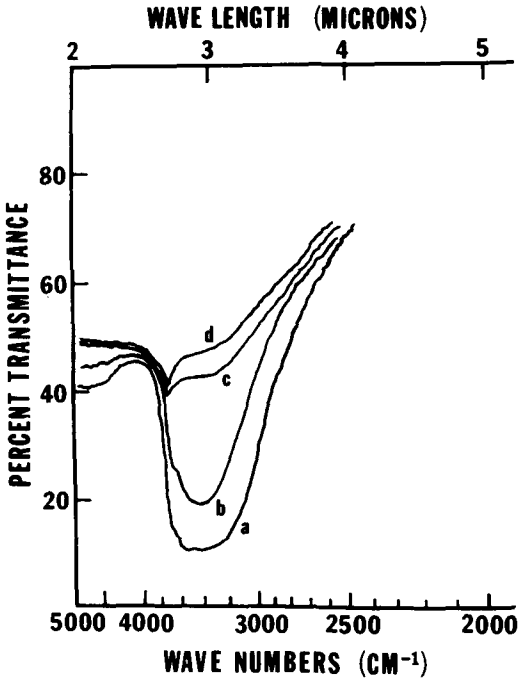

FIG. 2. Infrared spectra of Silica Gel G discs: (a) control, only physically adsorbed water was removed; (b) heated for 15 min at 300 C in vacuo; (c) heated for 15 min at 400 C in vacuo; (d) heated for 1.5 hr at 450 C or for 1.0 hr at 490 C in vacuo.

The characterization of amorphous silica gel surfaces has progressed considerably in recent years. It was found by chemical methods that for precipitated amorphous silicas with medium to wide pores and specific surface areas ranging from 200-500 m^2/g, the surfaces invariably contain a total of 4.6-5.2 silanol groups/100 Å^2 (13,22,23). Of these, 1.4 ± 0.1 groups exist as isolated non-hydrogen-bonded sites and the remainder mostly as hydrogen-bonding interacting pairs except for a small percentage of these as anomalous hydroxyl sites. It should be pointed out that regardless of differences in commercial source, the agreement among investigators on these values is excellent, particularly on the value of non-hydrogen-bonded sites. The existence of the isolated non-hydrogen-bonded hydroxyls is apparently due to the fact that, in the amorphous state, silicon atoms exposed on the surface are not fully saturated with hydroxyl groups as in the case of highly ordered crystalline silicates, but there is a random presence of siloxane groups as end groups preventing close contact of hydroxyl groups for hydrogen bonding. The reported value of 1.4 ± 0.1 groups/100 Å^2 for the non-hydrogen-bonded hydroxyls is very similar to our concentration of adsorbed linoleic acid on the surface (1.25 molecules/100 Å^2) obtained from the maximum adsorption in Figure 1.

With this agreement in mind, dehydration and infrared studies were undertaken to acquire definitive proof on this point. The removal of different types of surface hydroxyl groups by heating at various temperatures has been well documented. For example, it has been known that the physically adsorbed water, presumably hydrogen-bonded to the vicinal hydroxyl groups on the surface, could be successfully removed by evacuation at 150 C. Heating with evacuation between 200 C and 500 C ± 50 C leads to various degrees of dehydration of hydrogen-bonded hydroxyl groups and eventually all of this type of adsorption sites is eliminated. The concentration of non-hydrogen-bonded hydroxyls, 1.4 ± 0.1 groups/100 Å^2, however, remains constant at evacuation temperatures up to 600 C ± 50 C (13,14,22,24).

Using the available conditions for dehydration, various degrees of hydrogen-bonded hydroxyl sites were removed from silica gel surfaces and adsorption of linoleic acid from hexane on these surfaces was attempted. The results are summarized in Table II. In our dehydration experiments, it was found that the physically adsorbed water was successfully removed by heating at 130 C overnight, or at 150 C for 30 min in the ambient atmosphere, or at 180 C for 15 min with evacuation. The quantity of water removed as physically adsorbed water from Silica Gel G amounts to 4.2 molecules/100 Å^2. When the dehydration was not carried out prior to monolayer preparation, monolayer coverage dropped to about 92% of that of the dehydrated sample. This effect casts some doubt on the idea that water molecules adsorbed on silica gel surfaces are exclusively bound to the hydrogen-bonded hydroxyls (14,24). Upon complete removal of the physically adsorbed water, further loss of water is negligible until the temperature reached 200 C. The number of hydrogen-bonded sites lost, 3.65 and 3.36/100 Å^2 (reflecting slightly different heating conditions) shown in Table II, is somewhat larger than the values reported for the silica gel of similar types. This may indicate the number of hydrogen-bonded sites available in our silica gel or it might represent an inclusion of a small fraction of non-hydrogen-bonded hydroxyls beginning to dehydrate at these temperatures. This uncertainty also prevails over the interpretation of the resulting slightly lower monolayer coverages, 86.1% and 88.0% (Table II), on the adsorption study of these heat-treated samples. Thus, the departure from 100% coverage could represent either the

extent of loss of the non-hydrogen-bonded sites or the order of magnitude of fatty acid molecules bound to the hydrogen-bonded sites. The ambiguity existing on this point is not possible to resolve with the data presently available. In connection with the studies on monolayer coverage of the extensively dehydroxylated silica gel, it should be emphasized that specific surface areas (in m^2/g) are known to remain essentially constant up to about 700 C. Above this temperature, the surface area decreases sharply because of an overall sintering process caused by lattice diffusion and closure of cavities on the surface (22,24).

In an attempt to obtain additional evidence to support the idea that in samples heated at 450 C and 490 C, there was actually a total loss of hydrogen-bonded hydroxyls and use was made of infrared spectroscopy of silica gel. Self-supporting pressed discs of silica gel were used to avoid the scattering of the incident beam. Figure 2 shows the OH stretching absorption bands of silica gel discs heat-treated at various temperatures. Both Silica Gel G and H gave essentially the same results. It is known that the non-hydrogen-bonded isolated hydroxyls exhibit a sharp intense absorption at 3750 cm^{-1} and this merges into a very broad band of hydrogen-bonded adjacent hydroxyls centering at about 3550 cm^{-1} (13,14,25,26). As shown in Figure 2, upon heating, the component OH bands lose overall intensity and broadness especially on the lower wavenumber side. At 400 C, the bulk of the broad hydrogen-bonded OH band disappeared, and the sharp non-hydrogen-bonded band, which was present just as a shoulder in the control sample, became clearly discernible. The heat-treatment at a higher temperature at which all of the hydrogen-bonded sites are presumably eliminated (as indicated by the weight losses) further reduced the intensity and broadness of the hydrogen-bonded band but did not entirely eliminate the absorption in the 3500 cm^{-1} region as expected. Moreover, subsequent prolonged heating at 600 C failed to produce a further decrease in the intensity of this band. Therefore, a small amount of remaining absorption in the 3500-3600 cm^{-1} region is likely to be partly due to the absorption of the hydrogen-bonded internal or bulk hydroxyls centered at 3650 cm^{-1} (13,14) also indicated by a slight overall shifting of the absorption band toward shorter wave length. Hydrogen-bonded bulk hydroxyls are known to be completely removed only by heating to 700 C for 48 hr (27). It was suspected that the presence of moisture in the instrument and elsewhere might also be another cause of the remaining absorption in this region.

Therefore, the evidence from the dehydroxylation and infrared studies strongly suggests that, in adsorbed monolayers, fatty acid molecules are predominantly hydrogen-bonded to the isolated hydroxyl groups on the silica surface. The adsorption limits, therefore, are strictly governed by the availability of these surface sites rather than by other factors previously proposed by other investigators. Hockey and co-workers earlier had proposed that the adsorption of fatty acids on silica gel appears to be controlled solely by the aggregation of the solute in the bulk solution (27). However, as this work was in progress [a preliminary account has been published (28)], the same authors have abandoned the original hypothesis and briefly suggested a revised view also involving the isolated non-hydrogen-bonded silanol sites in the adsorption of fatty acids and methyl esters (29).

The monolayer of fatty acid-silica gel system with its presumed carboxyl end attachment to the silica gel and with the hydrocarbon chains more or less perpendicular to the surface, offers a somewhat defined system with which to begin the study of nature of non-enzymatic lipid autoxidations in membranes.

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Autoxidation of Fatty Acid Monolayers Adsorbed on Silica Gel: II. Rates and Products

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ABSTRACT

Unsaturated fatty acid monolayers on silica gel have been autoxidized, and the rate of fatty acid disappearance and products obtained from those membrane-like assemblies have been studied Fatty acid monolayers consisting of pure linoleic acid, linolelaidic acid, and oleic acid were autoxidized at 60 C. The rates of autoxidation of linoleic acid and linolelaidic acid monolayers followed by the disappearance of substrates are considerably faster than that in bulk phase, and the rates conform to apparent first order kinetics. Autoxidation of linoleic and linolelaidic acid monolayers, unlike bulk phase, produced only a small amount of diene conjugation, and the major products formed were identified as 9,10-epoxy and 12,13-epoxyoctadecenoic acid in roughly equal quantities. The epoxidation is stereospecific, with *cis* and *trans* olefins giving *cis* and *trans* epoxides, respectively. Oleic acid was autoxidized to only a small extent during 27 hr and produced no detectable amount of epoxide.

INTRODUCTION

The use of adsorbed monomolecular films as models for the study of non-enzymatic membrane lipid autoxidation has been attempted by several investigators. Honn and co-workers (1), using silica gel as the support for soybean oil, were the first to correlate the different rate of autoxidation to different ratio of substrate to solid support. Porter et al. (2) refined the experiment and demonstrated that the maximum rate of autoxidation was exhibited by linoleic acid to silica ratio close to that for a monolayer. Using the linoleic acid-silica gel system, Porter et al. also studied the effects of prooxidants and antioxidants on the rate of lipid autoxidation (2). Such an adsorbed system with a considerably lower ratio of fatty acid to silica than that of a monolayer has also been found to lower the rate of autoxidation of unsaturated fatty acids (3,4).

All the earlier rate studies carried out with monolayers (1,2,5,6) used oxygen uptake as a measure of autoxidation. In recent years, however, the apparent oxygen consumption by unsaturated fatty acids has been recognized to involve "oxygenation" (a complex formation process between double bonds of the fatty acid and oxygen) as well as autoxidation (7). True autoxidation apparently has a slower initial and continuing rate than does the oxygenation process. For these reasons, we have felt that the extent of monolayer autoxidation can be more appropriately followed by measuring the quantities of unchanged substrate extracted from the silica gel after oxidation.

Previous studies (1-6) also dealt sparingly with the products of lipid autoxidation in monolayers. A number of instances have been reported in recent years in which the membrane-like ordered arrangement has exerted a major influence over the course of a reaction so that the products formed differ completely from those normally obtained from bulk phase or solution (8,9). That such an influence, arising from the spatial arrangement of molecules, also exists in our system is indicated by a previous finding that, unlike bulk phase autoxidation, diene conjugation is formed only in small quantities in monolayers (10).

We undertook studies of monolayer autoxidation of unsaturated fatty acids commonly found in biomembranes with the emphasis on the major products of the reaction. It was our hope that the knowledge gained of the process of autoxidation with this simple model might lead to a rational approach to the understanding of non-enzymatic autoxidation of biomembranes. In this study, the reaction rates and products from linoleic acid, linolelaidic acid, and oleic acid monolayers adsorbed on silica gel are presented and a hypothesis advanced to elucidate the formation of major products from this system.

EXPERIMENTAL PROCEDURES

Materials and Methods

Linoleic, linolelaidic, and oleic acids purchased from Applied Science Laboratories, Inc. (State College, PA) are 99+% pure and were used directly after a purity check with thin

layer chromatography (TLC) and gas liquid chromatography (GLC). The important physical characteristics of both the Silica Gel G and H used have been reported elsewhere (11).

GLC was carried out using a Varian Aerograph Model 2100 equipped with flame ionization detectors and an electronic integrator (Infotronics Corp., Model CRS-11 HSB). GLC columns used were an 0.20 x 183 cm glass U-tube contining 3% OV-101 on 100/120 mesh Gas Chrom Q and an 0.20 x 90 cm tube containing 15% DEGS on 80/100 mesh Gas Chrom Q. Curve resolution of overlapping GLC peaks was carried out with a DuPont 310 Curve Resolver. Column chromatography was carried out using a 3.4 x 8.0 cm silicic acid column (J.T. Baker Chemical Co. Phillipsburg, NJ) prewashed with acetone, ether, and n-pentane and developed with pentane with increasing amounts of ether. Two column-volumes of each solvent mixture were used with collection of half column-volumes of eluate for each fraction. Mass spectroscopic analyses were performed on a Finnigan Model 300 quadrupole spectrometer coupled with a Varian Aerograph Series 1400 gas chromatograph with an 0.20 x 150 cm coiled column packed with 3% OV-1 on 60/80 mesh Gas Chrom Q. The methoxy trimethylsilyl derivatives of epoxides were obtained by first treating the epoxide with BCl_3 in methanol followed by a silylating reagent, TRI-SIL (Pierce Chemical Co., Rockford, IL) containing Me_3SiCl, $(Me_3Si)_2NH$, and pyridine. Infrared spectra were obrained with either Baird Associates Model B or Infracord Model 137 (Perkin-Elmer Corporation, Norwalk, CT) instruments, using CCl_4 as solvent. Ultraviolet (UV) spectra were recorded using 1 cm path quartz cells in a Cary Model 14 Spectrophometer. TLC was carried out using precoated Silica Gel G plates (0.25 mm thick, Analtech Inc., Newark, DE) with solvent system: petroleum ether-diethyl ether-acetic acid, 80:20:1. Visualization of spots was accomplished by spraying with 3% cupric acetate solution in 8.5% phosphoric acid with subsequent charring at 140 C. For preparative TLC, samples were spotted on prescored plates; one segment of the plate was broken off for spraying and charring to locate the spots, and the spots were scraped off and extracted with ether.

Preparation and Autoxidation of Monolayers

In a typical preparation, a solution of 0.531 g (0.00189 mole) of linoleic acid in 43 ml of hexane (Phillips Petroleum Co., Bartlesville, OK, pure grade) was poured on 2.004 g of Silica Gel G which had been dehydrated at 130 C overnight prior to coating. The mixture was stirred for 1 hour, allowed to settle for at least 5 min, and the supernatant was withdrawn as completely as possible. Evaporation of the hexane from the supernatant left 0.144 g of linoleic acid as residue, the remaining 0.386 g (0.00138 mole) was adsorbed by silica gel. The fatty acid-coated silica gel was first freed from the bulk of the solvent by a rotary evaporator and was then dried under high vacuum. The fatty acid-coated silica was flushed with nitrogen and protected from light as much as possible during the entire preparation.

The dry coated silica, about 0.220 g, was placed in a 125 ml Erlenmeyer flask with a stopper and was vigorously shaken in a 60 C incubator for the desired length of time. The amount of oxygen enclosed in the headspace of the flask was calculated to be at least ten times that required for autoxidation of all the linoleic acid present in the flask. After the incubation, the content was extracted three times with methanol. Although ether extracts less inorganic material from silica, the efficiency of recovery of linoleic acid from silica gel is only about 96-97% compared with 99-100% recovery by methanol. The combined methanol extracts were freed from the solvent and were esterified with diazomethane. To the resulting methyl ester, 2 ml of methyl stearate solution (usually about 200 mg in 25 ml of isooctane) was added as an internal standard, and the mixture, properly diluted, was then quantitated by GLC. Peak areas were obtained by an electronic integrator, and the relative responses of methyl stearate and substrate were determined by running mixtures containing known weights of methyl stearate and the substrate. No difference in autoxidation rate was observed in monolayers of linoleic acid supported on Silica Gel G or H.

An anaerobic incubation of linoleic acid monolayers was carried out by the following procedure. About 220 mg of acid-coated silica was placed in a test tube with a constriction and was evacuated to 0.1 mm Hg before it was filled with pure argon (Liquid Carbonic, regular grade, minimum guaranteed purity, 99.998%). The evacuation and refilling with argon were repeated four times before it was finally filled with 1 atm of argon and sealed at the constriction. A control tube was prepared in exactly the same way as the argon-filled except that a small hole was left in the tube. Both tubes (6.5 x 0.9 cm) were incubated and worked up as for the above aerobic runs.

Bulk Phase Autoxidation

Linoleic acid (about 35 mg) spread as a thin film on the bottom of a flask was left in a 60 C incubator-shaker for the desired length of time. The disappearance of linoleic acid was determined by esterification followed by addition of methyl stearate solution (as internal standard) to the entire contents and quantitation by GLC as in the monolayer autoxidation.

RESULTS

For most of the autoxidation studies, a monolayer coverage of 0.69 mmole of fatty acid per g of silica gel was selected mainly to reduce the amount of nonmonolayer adsorption resulting from the entrapped solution after decantation of supernatant solution. At this coverage, the linoleic acid from an equilibrium solution entrapped in a preparation of 2 g of silica gel causes multilayer deposit of only about 3% of the true monolayer adsorption. Although this particular ratio of fatty acid to silica amounts to binding of only 89% of the available isolated silanol sites on the surface (11), the coating throughout the surface is apparently uniform; a random sampling of portions of coated silica gave a mean deviation of ± 1% in fatty acid recoveries. The extent of reduction in coverage here is not likely to affect the observed rate of monolayer autoxidation, since we demonstrated previously that the rate of autoxidation remains constant throughout a wide range of linoleic acid to silica ratios, decreasing sharply only below 20 mg of linoleic acid per g of silica (12). Free fatty acids rather than methyl esters were used throughout the study since it was found that hydrolysis of esters occurs concomitantly with autoxidation (G.-S. Wu and J.F. Mead, unpublished observations).

The rates of autoxidation of monolayers of linoleic acid, linolelaidic acid, and oleic acid at 60 C are shown in Figure 1. The rate of monolayer autoxidation at room temperature, unlike that of bulk phase, was found to be appreciable. Even with precautions to minimize the exposure to air, the amounts of fatty acid extracted before incubation (as determined by GLC) are usually only 92 to 95% of the theoretical amount The role, if any, played by the metals in the silica gel has not been determined. The inclusion of $CaSO_4$ in Silica Gel G does not affect the autoxidation rate because essentially the same rates were obtained on Silica Gel G and H. In experiments of monolayer autoxidation, the amount of substrate recovered before the autoxidation is normalized to 100% to serve

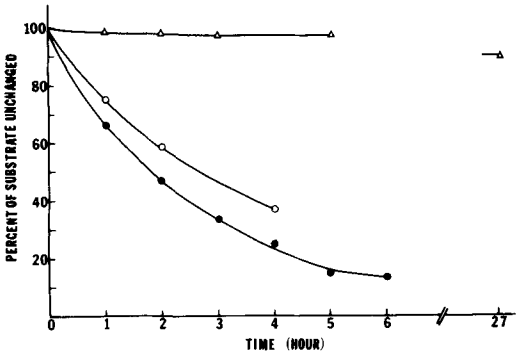


FIG. 1. Plot of percent of substrate unchanged vs. time (hr) incubated at 60 C. —●—●—, Linoleic acid; —○—○—, linolelaidic acid; and —△—△—, oleic acid.

as control at zero hour. As shown in Figure 1, in the silica monolayer system, for linoleic acid and its *trans* isomer, the autoxidation commences immediately without a detectable induction period. For linoleic acid, the rate of autoxidation in monolayers is considerably faster than that in bulk phase. For example, at 60 C, in the bulk phase, the unaltered linoleic acid is 90.5% after incubating for 6.5 hr and 23.1% after 16.5 hr; whereas in monolayers, the similar figures are 66.0% after 1 hr incubation and 13.1% after 6 hr incubation. The amounts of crude autoxidized material extractable from silica gel decreases gradually as the autoxidation progresses. For example, the weights recovered by extraction with methanol from linoleic acid monolayers are: for control, 98.2%; for 1 hr incubation, 96.1%; 2 hr, 94.5%; 3 hr, 90.5%; 4 hr, 85.8%; and 5 hr, 82.0%. This apparent loss of material is probably due to the presence of an increasing amount of unextractable polymeric material formed in the course of autoxidation. The monolayer autoxidation of oleic acid was found to be extremely slow. At 60 C, the alteration of the starting material was only 10% after 27 hr.

The exposure of adsorbed linoleic acid monolayers to air at 60 C for 3 hr leads to formation of at least four major products at the expense of 67% of the starting material. That these products are entirely different from those of bulk phase is evident from TLC of esterified total crude product from two sources (Fig. 2). The bulk phase products displayed in lane 1 for comparison were obtained by autoxidizing linoleic acid as a thin film at 60 C to about the same extent of conversion.

In the bulk phase, spot B, which is the major product at this stage of autoxidation, was identified as linoleic acid hydroperoxide based on

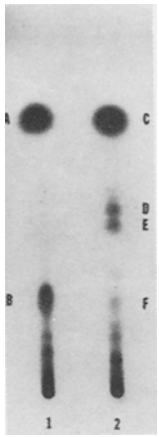


FIG. 2. Thin layer chromatograms of linoleic acid autoxidized at 60 C in bulk phase (lane 1) and in monolayer (lane 2). A and C, unchanged methyl linoleate; B and F, methyl linoleate hydroperoxide; D, methyl 12,13-epoxyoctadecenoate; E, methyl 9,10-epoxyoctadecenoate.

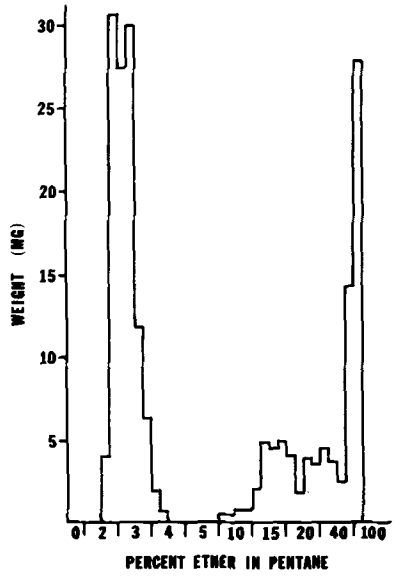


FIG. 3. Silicic acid column chromatogram of products from linoleic acid autoxidized for 3 hr at 60 C.

the following evidence: (a) spraying with potassium iodide solution gave a distinct yellowish brown spot which, on contact with starch solution, turned to blue; (b) the scraped and extracted material gave an absorption maximum at 232 nm, indicative of diene conjugation. In bulk phase autoxidation, at this stage, most of the diene conjugation formed is known to appear in hydroperoxides. The identification of the hydroperoxide spot in TLC immediately reveals the fact that in monolayer autoxidation, very little hydroperoxide is isolated. This conclusion is also supported by the results of UV absorption of the total crude product. In Table I, the amounts of conjugated diene (or hydroperoxide) in monolayer autoxidation, calculated from the intensities at 232 nm, are compared with those from bulk phase at two different stages.

The first indication that two of the major products in monolayers (spot D and E in Fig. 2) are structurally closely related epoxides came from an observation tht the Rf values (0.39 and 0.35) are similar to those of 9,10-epoxy stearates. Silicic acid column chromatography of the total autoxidized material (diazomethane treated) carried out for the purpose of isolating some epoxide is shown in Figure 3. On gradient elution with pentane-ether mixtures, the unchanged starting methyl linoleate as well as methyl stearate (added as internal standard for GLC runs) was eluted with 2-3% ether in pentane. This was followed by distinct fractions of epoxides eluted with 15% ether. 9,10-Epoxy stearates are known to be eluted from silicic acid columns with 7-10% ether in pentane

TABLE I

Formation of Diene Conjugation in Monolayers and Bulk Phase Autoxidation of Linoleic Acid at 60 C

Form of substrate	% of linoleic acid unchanged	% of diene conjugation in the mixture[a,b]	% of diene conjugation in the product[a,b,c]
Monolayers	66.0	7.8	22.9
Monolayers	33.4	4.9	7.4
Bulk	65.0	29.8	85.1
Bulk	23.1	33.8	44.0

[a]Calculated as linoleic acid hydroperoxide.

[b]Molar extinction coefficient of 23,300 was used for the estimation.

[c]Corrected for the amount of starting material in the mixture.

TABLE II

Relative Retention Times and Areas in GLC of Epoxides from Monolayer Autoxidation

Source	Epoxide isomers[a]	Retention times (min)	Yield (%)
Linoleic acid monolayers	9t 12c and 9c 12t	28.5	11
Linoleic acid monolayers	9c 12c and 9c 12c	31.5	89
Linolelaidic acid monolayers	9t 12t and 9t 12t	28.2	92
Linolelaidic acid monolayers	9c 12t and 9t 12c	33.0	8

[a]The underlines indicate the positions of epoxide, thus 9t 12c denotes *trans*-9,10-epoxy-*cis*-12-octadecenoate.

(13). The two distinct compounds resolved on TLC (Fig. 2, spots D and E) are not separable in column chromatography. The resolution attained is apparently only partial, the spot D being concentrated in the early half of the peak (with 15% ether) and spot E in the trailing half of the peak.

Small quantities of the two epoxides (about 1 mg each) (spots D and E) were eventually obtained by preparative TLC runs and were respotted on the plate to insure purity. Both compounds gave no detectable UV absorption except a low intensity peak at 275 nm in both cases, suggestive of ketodienoic esters. This contamination was estimated to be in spot D, 1.5% and in spot E, 2.0% of the total weight. (Rf value of 9-oxostearate is 0.37.) Infrared spectra of both epoxides resemble those of *cis*- and *trans*-9,10-epoxy stearate closely in overall spectrum and ester carbonyl absorption at 5.71 μ, except that a discernible *cis* olefinic CH absorption at 3.25 μ is present in both compounds from monolayer oxidation. An absorption band assigned to the epoxide ring at 8.00 μ (14) is barely noticeable in 9,10-epoxy stearate and our two epoxides.

Gas chromatography-mass spectrometry of saturated and unsaturated long chain epoxy acids has been reported by several investigators (15-17). Mass spectra of saturated epoxides are extremely simple, the high intensity ions being those from fragmentation of C-C bonds α to epoxide ring. The presence of double bonds in the chain, however, as also observed by others (16,17), decreases the simplicity of the spectra drastically. The spectra of two epoxides from autoxidation were found to be complex and difficult to interpret, except that a low intensity molecular ion at 310 is clearly visible in both cases. Among several known chemical methods to modify the epoxy group in unsaturated compounds, we found that solvolysis in methanol and subsequent conversion to trimethylsilyl ether is straightforward, and the resulting spectra confirm the assigned structures. From the less polar epoxide (spot D in Fig. 2), the following major ions, m/e 173, $CH_3(CH_2)_4CHOSi(CH_3)_3$; m/e 217, $CH_3(CH_2)_4CH(OCH_3)CHOSi(CH_3)_3$; m/e 299, $(CH_3)_3SiOCHCH_2CH{=}CH(CH_2)_7COOCH_3$; and m/e 195, $OHCCH_2CH{=}CH(CH_2)_7CO$, characteristic of methyl 12,13-epoxy-9-octadecenoate (16,17), were obtained. From the more strongly adsorbed epoxide (spot E in Fig. 2), the major ions are m/e 213, $CH_3(CH_2)_4CH{=}CHCH_2CHOSi(CH_3)_3$; m/e 259, $(CH_3)_3SiOCH(CH_2)_7COOCH_3$; m/e 303, $(CH_3)_3SiOCHCH(OCH_3)(CH_2)_7COOCH_3$ and m/e 155, $OHC(CH_2)_7CO$; thus, spot E is positively identified as methyl 9,10-epoxy-12-octadecenoate. The ratio of 9,10-to 12,13-epoxide determined by comparing the peak heights of ions, m/e 213 or m/e 217, in pure 9,10- or 12,13-epoxide with that of total unfractionated epoxide from autoxidized material (3 hr run) is about 1.3.

An attempt was also made to resolve the *cis*- and *trans*-unsaturated epoxides by GLC. *Cis*- and *trans*-unsaturated epoxidies structurally similar to ours have been resolved partially by Gunstone and Schuler using a DEGS column (18). With a DEGS column, we found that the saturated pair, *trans*- and *cis*-9,10-epoxystearate, gave retention times of 27.4 min and 29.2 min, respectively. Isomers of unsaturated epoxides from autoxidation, although not completely resolved, were considerably better separated than their saturated counterparts. With the aid of a curve resolver, epoxide isomers from linoleic acid monolayers as well as linolelaidic acid monolayers were successfully re-

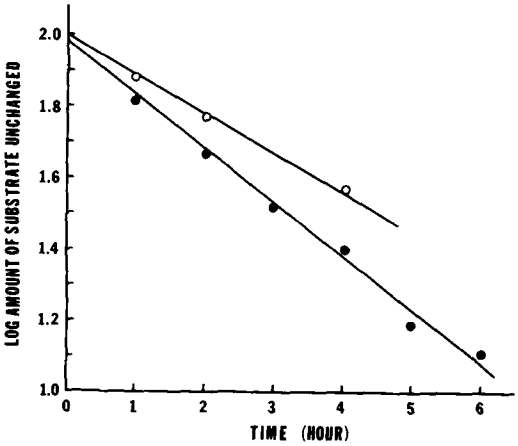


FIG. 4. Plot of logarithm of unchanged substrate vs. time (hr) incubated at 60 C. —●—●—, Linoleic acid; and —○—○—, linolelaidic acid.

solved and quantitated as shown in Table II. The absolute quantities of epoxides produced increase slightly from 1 to 4 hr of incubation (with the maximum amount being at 4 hr) and then decrease with further incubation. With 3 hr incubation, the epoxide isolated amounts to ca. 30% of the total product.

The monolayer autoxidation of linolelaidic acid produces epoxides in yield comparable to that from linoleic acid under identical conditions. The epoxides formed in this case are predominantly *trans*, whereas linoleic acid gave mostly *cis* epoxides (Table II). Oleic acid monolayers gave no detectable amount of epoxide at any stage of autoxidation, even after incubation for 27 hr (Fig. 1).

For establishing that the oxygen atom incorporated in the epoxide ring actually comes from atmospheric oxygen, an attempt was made to carry out the monolayer autoxidation in pure argon. Linoleic acid monolayers sealed in an ampule with 1 atm of pure argon were incubated for 130 min at 60 C along with a control which was open to the air. The control gave 42% of unchanged linoleic acid, a value close to that observed in the rate study (Fig. 1), whereas from the argon-filled sample, 93% of the starting material was recovered.

DISCUSSION

The rate of autoxidation of linoleic acid monolayers, as shown in Figure 1, differs from that of bulk phase in several major aspects: (a) there is no lag period in monolayers, (b) the rate of disappearance of linoleic acid is considerably faster in monolayers than in bulk, and (c) the rate of autoxidation is clearly first order in linoleic acid as shown by an excellent linear relationship between the log of amount of remaining linoleic acid and time of incubation (Fig. 4). Other polyunsaturated fatty acids adsorbed on silica at a concentration considerably less than a monolayer also exhibit this first order dependence on the substrate (4). Simple first order kinetics implies, among other things, that the overall reaction is not autocatalytic in nature as in bulk phase, in which the overall kinetic behavior is complex and obeys none of the simple kinetic laws (19).

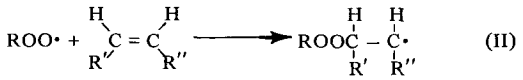
The role metal in the silica gel is playing in producing the differences in monolayer and bulk phase autoxidation is poorly understood. In the first place, metal analysis gives an average metal content and does not answer the question important here of how much of the metal resides on the surface of the silica gel. Second, there is no evidence that the middle of the fatty acid chains where the autoxidation is occurring comes into contact with the surface in the monolayer system. Evidence (2) suggests that a lower metal content slows the rate of autoxidation on silica gel, but whether the metal affects the initiator concentration, promotes a radical chain propagation step, or is involved with terminator steps in our particular system is unknown. Research in this area is continuing.

Long chain epoxy acids have been observed as one of the minor products in bulk phase autoxidation of oleic and linoleic acids but were never isolated in major quantities comparable to that from monolayer autoxidation. Pure linoleic acid hydroperoxide under iron catalyzed decomposition has been shown to produce some unsaturated keto and hydroxy epoxides among a series of complex products (20). Any mechanism postulated for this process would have to account for the following typical observations: (a) an apparently simple kinetic behavior; (b) small amounts of conjugate diene and hydroperoxide formed at any stage of oxidation; (c) a stereospecific process of epoxidation; (d) almost equal involvement of both 9,10- and 12,13-unsaturations; (e) no epoxidation in a monoene monolayer; and (f) the silica surface-related specific orientation of reactants (11).

Considering all the facts, the formation of epoxide in this system could best be rationalized as involving an addition of performed peroxy radicals to the double bonds of the neighboring fatty acid (equation II). The occurrence of this type of addition as an alternative to the usual allylic hydrogen abstraction, although of minor importance, has been recognized in bulk phase autoxidation (21). In a

classical allylic oxidation of a simple 1,4-diene system, the peroxy radicals produced in reaction (I) proceed exclusively to abstract allylic hydrogen atoms at the chain-carrying step to form 9- and 13-hydroperoxides. The process of addition to double bonds, however, is made highly competitive with

$$R\cdot + O_2 \longrightarrow RO_2\cdot \qquad (I)$$

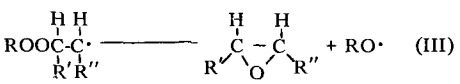


(II)

the abstraction mechanism by the presence of special structural features, such as conjugated dienone or diene ester (22), unreactive allylic hydrogen atoms (21), and a combination of methyl group and a terminal double bond (21).

In a monolayer autoxidation, the required activation or lowering of the activation energy for the addition process might be partially accomplished by having the reactants in the adsorbed state. In silica gel supported linoleic acid monolayers, there is strong suggestive evidence that the fatty acid molecules are predominantly hydrogen-bonded to the isolated surface silanol groups at the carboxyl ends (11). The hydrocarbon chains therefore protrude from the silica surface vertically except for bends or kinks caused by unsaturation. Consequently, the hydrocarbon chains could be aligned in such close proximity that only the addition reaction is favorable. It is understandable that the addition will be rather indiscriminate towards the two double bonds in linoleic acid.

For β-peroxylalkyl radicals formed by the addition of peroxy radicals to olefins, it has been found that the greater the stability of the radical, the more likely it is to react with oxygen rather than undergo unimolecular decomposition (23). For the resulting radical in reaction II, no resonance stabilization could be expected except perhaps a small degree through hyperconjugation; unimolecular decomposition would probably be a dominant fate. To account for the stereospecific epoxidation, this reaction, as shown in equation III, has to be faster than the rotation of a single bond, or reactions II and III must be a concerted process, from which a single bond is never formed at any stage.



(III)

The rate of epoxide formation through peroxy radical addition has been noted previously to depend on the structure of the alkene (24). In the present study, the difference in rate of disappearance of 9,12-*cis,cis*- and 9,12-*trans,trans*-octadecadienoic acid could be attributed to the steric effect of the two unsaturated systems, *cis,cis*-unsaturation being more accessible to the incoming peroxy group than *trans,trans*-unsaturation. In monolayers of monounsaturated fatty acid, such as oleic acid, the absence of epoxide formation would appear to be because of the lack of peroxy radicals.

One result inconsistent with the addition mechanism discussed is the scarcity of diene conjugation found in the product. The sequence of the reactions given in equations I to III requires that the ratio of epoxide to conjugated alcohol be equal to close to unity. In our case, the quantity of total diene conjugation found (including hydroperoxide and conjugated alcohol) amounts to only a small fraction of epoxide formed except at the early stage. A reasonable assumption is that the conjugated diene alkoxy radicals give rise to products not containing the diene system either by polymerization or cleavage into smaller fragments. This assumption is partly supported by the observation that, from polymeric products (fractions eluted with 40 and 100% ether in the column chromatography of total crude product, Fig. 3), a medium intensity hydrogen-bonded OH absorption band was recorded in IR.

The origin of the differences in products and reaction kinetics between monolayer and bulk phase autoxidation is presently unknown, although it seems reasonable to presume that it has its basis in the silica gel surface and/or the orientation of fatty acids on the surface. Studies on several monolayer systems with the elucidation of the mechanism of epoxide formation as an immediate aim are in progress in this laboratory. The elucidation of this mechanism in monolayers is important from the point of view of understanding the nature of nonenzymatic autoxidation in biomembranes; as it turns out, it also might mimic the epoxidation of the iron-based mixed-function oxygenases. There has been considerable interest and speculation concerning the epoxidation by these enzymes in recent years (25).

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SHORT COMMUNICATION

Influence of Trichloroacetic Acid-Phosphotungstic Acid on the Thin Layer Chromatographic Mobility of Gangliosides

ABSTRACT

Trichloroacetic acid (TCA)-phosphotungstic acid (PTA) precipitation has been used as a faster procedure than dialysis for the isolation of gangliosides, but the TCA-PTA treatment causes striking abnormalities in the thin layer chromatographic mobilities of the gangliosides. However, a normal chromatographic pattern can be restored by treating the precipitated gangliosides with the tetrasodium salt of ethylenediamine tetraacetic acid followed by dialysis. Hence, TCA-PTA treatment does not appear to cause artifacts or hydrolysis of the gangliosides.

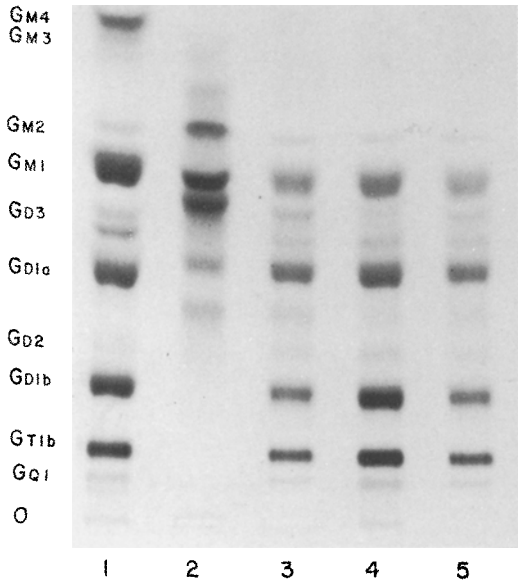

FIG. 1. Thin layer chromatogram of human brain gangliosides prepared by different methods (see text for details). Lane 1, standard (normal human white matter); 2, TCA-PTA precipitation; 3, TCA-PTA precipitation + EDTA + dialysis; 4, dialysis only; 5, EDTA + dialysis. The plate was developed by one ascending run in chloroform-methanol-water (60:35:8, v/v/v) containing 0.02% $CaCl_2 \cdot 2H_2O$. The bands were visualized by resorcinol spray and labeled according to the nomenclature system of Svennerholm (9).

INTRODUCTION

Precipitation of gangliosides with trichloroacetic acid (TCA)-phosphotungstic acid (PTA) has been used for the purification of gangliosides as an alternative to dialysis (1). TCA-PTA precipitation is particularly useful for more rapid determination of tissue total ganglioside content because the 3 day dialysis step is eliminated (2), and in metabolic studies, because the precipitation prevents the contamination of gangliosides with nucleotide sugars (1). However, alterations in the thin layer chromatographic mobility of gangliosides are known to occur after precipitation with TCA-PTA (3,4). Mestrallet et al. (4) suggested that TCA-PTA treatment of gangliosides may induce artifacts or expose structural differences among some of the gangliosides. In this study, we show that the treatment of TCA-PTA precipitated gangliosides with the tetrasodium salt of ethylenediamine tetraacetic acid (EDTA) followed by dialysis can restore a normal chromatographic pattern.

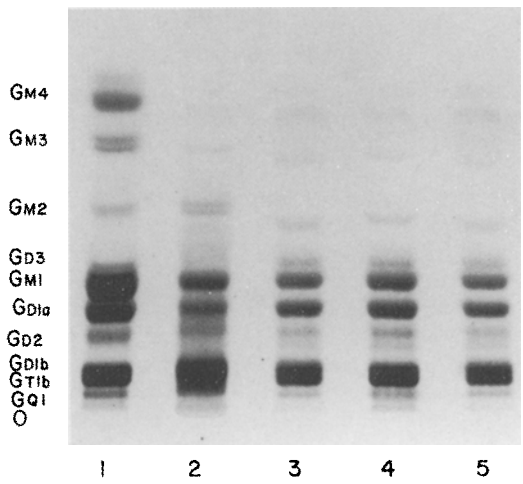

FIG. 2. Thin layer chromatogram of human brain gangliosides prepared by different methods. All the conditions are the same as shown in Figure 1 except that this plate was developed by one ascending run in chloroform-methanol-2.5 N aqueous ammonia (60:35:8, v/v/v).

MATERIALS AND METHODS

Gangliosides were extracted from a 1.757 g sample of normal human cerebral cortex by homogenization in 10 vol of chloroform-methanol (1:1, v/v). The tissue residue was washed once with the same solvents and enough chloroform was added to the system to give chloroform-methanol (2:1, v/v) concentration. The gangliosides were partitioned into the upper phase with the addition of 0.2 vol of water, and the lower phase was washed once with the "pure solvents upper phase" (5). The combined upper phases were evaporated under vacuum almost to dryness and then resuspended in 5 ml of distilled water. Four 1 ml samples of the aqueous upper phase were treated by one of the following methods: (a) TCA-PTA precipitation (1), (b) TCA-PTA precipitation as above then the addition of 0.5 ml EDTA .Na_4 (0.5M) followed by dialysis against water for 3 days at 4 C, (c) dialysis only, and (d) addition of EDTA followed by dialysis. After dialysis, the gangliosides were lyophilized and redissolved in chloroform-methanol (2:1, v/v). The amount of ganglioside N-acetyl-neuraminic acid (NANA) in each sample was determined by the resorcinol method (6) as modified by Miettinen and Takki-Luukkainen (7) and Suzuki (8). Samples, prepared by each of the four methods above, which contained ca. 15 μg of ganglioside NANA were spotted on Silica Gel 60 (Merck) plates (0.25 mm) and developed as shown in Figures 1 and 2. Analytical grade TCA and PTA were purchased from Fisher Scientific Company and Mallinckrodt Chemical Works, respectively.

RESULTS AND DISCUSSION

A normal chromatographic distribution of gangliosides was obtained after dialysis of the Folch upper phase (Fig. 1 and 2, lane 4); The same ganglioside pattern was obtained after treatment of the Folch upper phase with EDTA followed by dialysis (lane 5). Hence, EDTA alone had no marked influence on the normal chromatographic distribution of gangliosides. However, TCA-PTA treatment of gangliosides caused noticeable changes in ganglioside migratory behavior (lane 2), especially on the plate developed in the neutral solvent that contained $CaCl_2$ (Fig. 1). It appears that different solvent systems can significantly affect the migration of TCA-PTA precipitated gangliosides. The abnormalities induced by TCA-PTA treatment, however, were not permanent since a normal pattern could be restored after treatment of the precipitated gangliosides with EDTA followed by dialysis (lane 3). Seyfried (2) has shown that dialysis alone of TCA-PTA precipitated gangliosides was unable to completely remove the TCA-PTA induced chromatographic abnormalities. Since treatment with EDTA and dialysis restored the normal ganglioside pattern, it is unlikely that the TCA-PTA induced changes in ganglioside mobility were due to artifacts or from TCA-PTA accentuation of molecular differences in the structures of certain gangliosides. The abnormalities may result from ionic interactions of tungsten salts with some of the gangliosides. We suggest that EDTA removes the influence of TCA-PTA on gangliosides by converting the gangliosides to Na salt forms. It is also possible that TCA-PTA treatment causes the formation of inner esters (10) that may be removed by the mildly basic EDTA.

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LETTER TO THE EDITOR

A Convenient Synthesis of Dialuric (5-Hydroxybarbituric) Acid

Sir: In 1950 Rose and Gyorgy observed that red blood cells from animals depleted of vitamin E underwent extensive hemolysis when incubated in a dilute solution of dialuric acid (1). This test is widely used as an aid in evaluating nutritional vitamin E status. In the past several years, the few commercial suppliers of dialuric acid have ceased production of this compound presumably because of the low demand. This disappearance of the compound from the marketplace has handicapped investigators in the vitamin E field. The synthesis of dialuric acid has usually required special apparatus to prevent its ready oxidation by air or has utilized noxious reagents such as hydrogen sulfide and carbon disulfide (2,3). We describe here a simplified synthesis that requires a minimum of special equipment and involves no noxious chemicals. The procedure is similar to that described by Chang and Wu (4), but utilizes a more commonly available starting material.

PROCEDURE

Alloxan monohydrate (Eastman Organic Chemicals, No.1722, Rochester, NY) 5 g (33 mmol), platinum oxide (Adams catalyst, 0.5 g), methanol (30 ml), and water (20 ml) were shaken in a 250-ml hydrogenation flask under hydrogen at room temperature and pressure to absorption of one molecular equivilent (about 750 ml) during ca. 2 hr (absorption had nearly ceased by this time). The mixture was heated on the steam bath (until the colloidal suspension had flocculated) and filtered (light suction) through a one-half inch layer of Filtercel (Celite Analytical Filter-Aid, Johns Manville Co., New York, NY) on filter paper or sintered glass. Warm, 50% aqueous methanol, about 50 ml, was used to wash the catalyst and Filter-cel. Evaporation of the filtrate to dryness in a 70 C water bath (water-pump suction) left a residue which was triturated in methanol (about 25 ml). Filtration and drying in an evacuated desiccator gave 3.6 g (80%) of off-white dialuric (5-hydroxybarbituric) acid, mp 218-220 C (with decomposition). Pure white material was obtained by dissolving the product (1 g) in boiling water (30 ml), filtering the hot solution (filter paper, gravity or light suction), concentrating the filtrate on the hot plate to 15-20 ml, and cooling overnight at 5 C; yield 0.9 g. Heated in an open capillary, it became pink at 190 C, sintered at 220 C, but did not melt to 230 C (cf. ref. 2). It proved to be identical by infrared and mass spectrometry, M^+ 144 (molecular weight) and melting phenomena to authentic material. A bioassay by the vitamin E deficient rat red blood cell hemolysis test (1) gave results similar to those obtained with an authentic, commercial sample of dialuric acid.

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Identification of Positional Isomers of Lysophosphatides in Butter Serum

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ABSTRACT

The separation of individual classes of phospholipids by two-dimensional thin layer chromatography revealed the presence of lysophosphatides (monoacyl glycerophosphoryl compounds) in minor quantities in butter serum. The naturally occurring lysophosphatidyl ethanolamine (LPE) and lysophosphatidyl choline (LPC) were prepared by the combined use of a diethylaminoethyl (DEAE) cellulose column and thin layer chromatography. Gas chromatographic analysis of methyl esters of LPE and LPC showed that both lysophosphatides contained, in addition to saturated fatty acids, considerable quantities of unsaturated fatty acids. A comparison of the fatty acid pattern of naturally occurring LPE and LPC with that of LPE and LPC prepared by phospholipase A hydrolysis of phosphatidyl ethanolamine (PE) and phosphatidyl choline (PC) suggested that both the 1- and 2-forms of positional isomers were present. Hydrolysis of naturally occurring LPE and LPC with phospholipase C, and separation of the monoglycerides formed, confirmed the occurrence of two positional isomers of lysophosphatides.

INTRODUCTION

Bovine milk contains 0.03-0.04% phospholipids which are present in the milk serum (1,2) and in the membrane surrounding the milk fat globule (3-5). The milk phospholipids, though minor constituents of milk, play a very important role in the stability and oxidation of fat (6-8). Several extensive investigations have been carried out on bovine milk phospholipids. The distribution of these phospholipids and their fatty acid composition, as well as the distribution of the fatty acids between the 1- and 2-positions of PE and PC, are now fairly well established (9-13).

The presence of lysophosphatidyl ethanolamine (LPE) and lysophosphatidyl choline (LPC) in small amounts in bovine and other mammalian milks has also been reported (12,13). However, no information is available on the chemical structure of lysophosphatides from mammalian milks and this might be attributed to their minor concentration and problems in their isolation. This paper describes the isolation, the analysis of fatty acid composition, and the determination of the presence of two-positional isomers of lysophosphatides in butter serum.

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EXPERIMENTAL PROCEDURES

Extraction of Lipid Materials

Butter serum obtained from the fresh, unsalted butter was used as a convenient source of raw material from which milk phospholipids could be isolated and fractionated. Butter serum was frozen as a thin layer in polyethylene bags and freeze-dried. The lyophilized material was then extracted with chloroform-methanol (2:1, v/v) in the ratio of 20 volumes of solvent per gram of serum powder for 2-3 hr at 5 C under a nitrogen atmosphere. The mixture was filtered, and the filtrate was washed with 0.2 volume of distilled water to remove nonlipid material. The chloroform layer containing lipid material was recovered, dried over anhydrous sodium sulfate, and the solvent was evaporated to dryness in vacuo at <35 C using a rotary evaporator. The lipid materials were transferred in chloroform into a volumetric flask.

Two-dimensional Thin Layer Chromatography (TLC)

Two-dimensional TLC of the polar lipids was carried out on 20 x 20 cm chromatoplates of silica gel impregnated with sodium carbonate (14). A slurry was prepared by mixing thoroughly one part of silica gel without binder to two parts of 0.01M aqueous solution of sodium carbonate. The slurry was spread on clean glass plates to a thickness of 500 μ. The plates were allowed to air-dry and then activated for 2 hr at 110 C prior to use. An aliquot of lipid materials in chloroform was applied to the plate as a spot with a microsyringe. Usually the amount of lipid material was such that the lipid phosphorus was present in quantities of 10-15 μg. The plates were developed in the first direc-

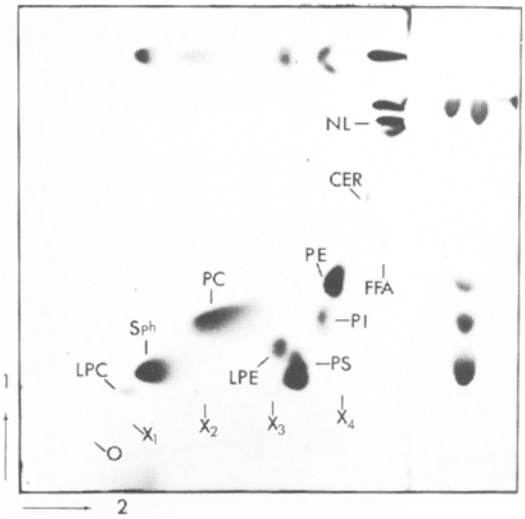

FIG. 1. Two-dimensional TLC chromatogram of milk phospholipids obtained from butter serum. Plates were developed in the first direction with chloroform-methanol-28% (w/v) ammonia-water (65:35:5:2.5, v/v) and in the second direction with chloroform-methanol-butanol-acetic acid-water (90:60:40:20:15, v/v). Abbreviations: NL, neutral lipids; CER, cerbroside; FFA, free fatty acid; PE, phosphatidyl ethanolamine; PC, phosphatidyl choline; Sph, sphingomyelin; PI, phosphatidyl inositol; PS, phosphatidyl serine; LPE, lysophosphatidyl ethanolamine; LPC, lysophosphatidyl choline; X, unidentified; O, origin.

tion with chloroform-methanol-28% (w/v) ammonia-water (65:35:5:2.5, v/v) and in the second direction with chloroform-methanol-butanol-acetic acid-water (90:60:40:20:15, v/v). Between the two runs, the plates were dried for about 15 min in a nitrogen atmosphere. After the TLC separation, all individual phospholipid spots were identified on the basis of their characteristic chromatographic properties relative to the standard reference lipids (Applied Science Laboratories, Inc., State College, PA) and their reactivity to spray reagents. Phospholipids containing primary amino groups were detected by spraying with ninhydrin solution. Choline-containing lipids were detected by the Dragendorff reagent modified by Munier and Macheboeuf (15). Anthrone reagent was used to detect sugar-containing lipids. A typical separation of phospholipids by two-dimensional TLC is shown in Figure 1. For lipid phosphorus analysis, the various phospholipid-containing spots and appropriate blanks were individually scraped off and placed in Pyrex test tubes. After digestion of the lipid material with perchloric acid, the phosphorus content was determined colorimetrically, as described by Eng and Noble (16).

Fractionation of Phospholipids

Preparation of a diethylaminoethyl (DEAE) cellulose (Matheson, Coleman and Bell, Norwood, OH) column and separation of phospholipids by column chromatography were performed as described by Rouser et al. (17). Briefly, about 300 mg of lipid dissolved in 5 ml chloroform were applied on the column (20 cm high, 2.5 cm ID) and washed into the column with three 10 ml portions of the same solvent. The following series of solvents were used to elute the phospholipid fractions. Fraction 1, containing the neutral lipids, was eluted with 500 ml chloroform. Fraction 2, containing cerebrosides, phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline, was eluted with 440 ml of chloroform-methanol (9:1, v/v). Fraction 3, containing phosphatidyl ethanolamine, was eluted with 550 ml of chloroform-methanol (7:3, v/v). Fraction 4, containing the lysophosphatidyl ethanolamine, was eluted with 500 ml of chloroform-methanol (1:1, v/v). The fractionation beyond the elution of LPE was not continued since the isolation of PC, LPC, PE, and LPE was the main object of this column chromatography. The solvent from each fraction was evaporated to dryness under vacuum, and the residue was redissolved in small portions of chloroform. Individual compounds were further purified by TLC.

Phospholipase A (EC 3.1.1.4) Hydrolysis of Phosphatidyl Choline (PC) and Phosphatidyl Ethanolamine (PE)

Phospholipase A hydrolysis was carried out under the conditions described by Nutter and Privett (18) using King Cobra venom (*Ophiophagus Hannah*) (Sigma Chemical Company, St. Louis, MO). An amount of 5-10 mg of purified PE or PC was dissolved in 3 ml of diethyl ether, to which was added 1 ml of 1M tris buffer (pH 7.5) containing 10^{-2}M calcium chloride and 5 mg snake venom. The reaction was carried out at room temperature in an atmosphere of nitrogen and stirred by a magnetic stirrer. After 6 hr of incubation, the reaction mixture was extracted with chloroform-methanol (2:1, v/v), and the lipid materials recovered were separated by TLC with chloroform-methanol-water (70:30:3, v/v). The free fatty acid and lysophosphatide bands were visualized with 2′,7′-dichlorofluorescein under UV light and scraped off into ampules for esterification. Fatty acid methyl esters were prepared, and the recovered methyl esters were subjected to gas liquid chromatography (GLC) analysis.

Phospholipase C (EC 3.1.4.3) Hydrolysis of LPE and LPC

The hydrolysis of purified lysophosphatides was carried out in an incubation medium consisting of 1 ml of 1M tris buffer (pH 7.2) mixed with an equal volume of diethyl ether and 5 mg of phospholipase C from *Bacillus cereus* (Laboratory Park, Chagrin Falls, OH) (19). Incubation was conducted for 6 hr under the same environmental conditions as the phospholipase A hydrolysis. The reaction mixture was extracted with diethyl ether, and the monoglycerides obtained were chromatographed as a spot along with standard 1-monopalmitin and 2-monostearin mixtures (Applied Sciences Laboratories, Inc.). The monoglycerides were separated with chloroform-acetone (88:12, v/v) on a boric acid-impregnated plate (20). Lipid spots were detected by spraying with 50% sulfuric acid and subsequent heating of the plate in the oven at 110 C.

Analysis of Fatty Acid Methyl Esters

The isolated PE, PC, LPE, and LPC were transesterified with 5% (v/v) solution of sulfuric acid in methanol in sealed glass ampules at 90 C for 2 hr. After phospholipase A hydrolysis of PE and PC, lysophosphatide bands developed on TLC plates were scraped off into glass ampules, and methyl esters were prepared by appropriate treatment with 5% sulfuric acid in methanol as described above. Free fatty acid bands on the TLC plates were scraped off into glass ampules, to which was added a small amount of benzene to wet the silica gel completely, and the free fatty acids were esterified with 2 ml of 14% boron trifluoride-methanol solution at 100 C for 10 min (21). The methyl esters prepared were dissolved in petroleum ether and stored under nitrogen at -10 C. For analysis, the solvent was evaporated under a stream of nitrogen, the methyl esters redissolved in carbon disulfide, and analyzed with an F & M model 402 gas chromatograph equipped with dual columns and dual flame ionization detectors. The glass columns (10 ft x ¼ in. OD) were packed with 60-80 mesh firebrick support coated with 15% (w/w) diethylene glycol succinate polyester. Routine analysis was performed isothermally at 185 C. Peak identifications were based on relative retention times relative to methyl stearate and comparison with a known standard methyl ester mixture (Applied Science Laboratories, Inc.). Relative peak areas were measured by the disc integrator (Disc Instrument Inc., Santa Ana, CA).

TABLE I

Composition of Phospholipids from Butter Serum[a]

PE[b]	PC	PS	PI	Sph	LPE	LPC
(% of total lipid phosphorus)						
27.3	29.1	13.4	2.5	25.6	1.2	0.9

[a]Results are the average of three determinations.
[b]Abbreviations: See Figure 1.

RESULTS

The separation of individual classes of phos-

TABLE II

Fatty Acid Composition of Natural Lysophosphatides and Positional Distribution of Fatty Acids of Phosphatidyl Ethanolamine and Phosphatidyl Choline[a]

Fatty acid[b]	PE[c]			Natural LPE	PC			Natural LPC
	1	2	Total		1	2	Total	
12:0	0.4	0.7	0.3	0.3	0.3	0.8	0.5	1.1
13:0	0.3	0.1	—	—	—	0.2	—	2.0
14:0	1.9	1.2	1.7	2.9	5.7	12.4	8.9	8.4
15:0	0.6	0.3	0.5	0.7	1.5	2.0	1.6	1.8
16:0	20.4	8.1	14.0	13.1	46.5	28.1	37.6	31.4
16:1	1.4	2.3	1.5	3.6	1.3	2.4	2.0	5.2
17:0	0.6	0.3	0.4	0.3	0.7	0.2	0.5	0.7
18:0	26.6	3.1	13.5	18.7	16.9	4.1	10.2	12.0
18:1	45.0	61.3	54.2	51.8	25.0	40.2	32.9	30.9
18:2	2.8	19.2	12.1	8.3	2.2	7.8	4.8	6.0
18:3	—	3.3	1.7	0.4	—	1.8	1.0	0.6
Sat.	50.8	13.8	30.4	36.0	71.6	47.8	59.3	57.4
Unsat.	49.2	86.1	69.5	64.1	28.5	52.2	40.7	42.7

[a]Expressed as average mole percent of three determinations.
[b]Number of carbon atoms: number of double bonds.
[c]Abbreviations: See Figure 1.

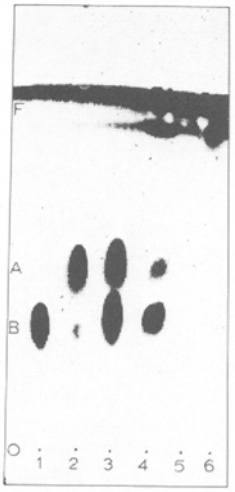

FIG. 2. Separation of isomeric monoglycerides on a boric acid-impregnated Silica Gel G TLC plate (250 μ thickness). The plate was developed with chloroform-acetone (88:12, v/v) and charred with 50% sulfuric acid for visualization. 1. 1-Monopalmitin. 2. 2-Monopalmitin. 3. Mixture of 1- and 2-Monopalmitin. 4. Monoglycerides obtained from natural LPC by phospholipase C hydrolysis. 5. The ether extract from a phospholipase C incubation without natural LPC. 6. The ether extract from an incubation of natural LPC without the enzyme. F: Solvent front, unidentified impurities present; A: 2-Monoglyceride; B: 1-Monoglyceride; O: Origin.

pholipids from butter serum was achieved by two-dimensional TLC (Fig. 1). The distribution of phospholipids given in Table I showed that PE, PC, PS, PI, and sphingomyelin are the normal constituents of milk phospholipids. Small amounts of lysophosphatides, LPE and LPC, were also present. The total fatty acids and specific distribution of the fatty acids in the 1- and 2-positions of PE and PC and the fatty acid composition of natural LPE and LPC, are shown in Table II. The fatty acid composition of PE exhibited a high level of unsaturation with oleic acid comprising about 54% of the total fatty acids. Data obtained on PE indicated that about 86% of the fatty acids in the 2-position were unsaturated, compared with 49% in the 1-position. Oleic acid was the principal fatty acid in both positions and constituted 45% and 61% in the 1- and 2-positions, respectively. The higher proportion of unsaturation in the 2-position was accounted for by the preferential presence of linoleic and linolenic acids, as well as oleic and palmitoleic acids. The stearic acid found in PE was mostly in the 1-position, while palmitic acid accounted for 20% and 8% of the acid component present at the 1- and 2-positions, respectively. Stearic and palmitic acids were the fatty acids responsible for the saturation of the 1-position. Myristic acid was found largely in the 1-position in PE.

The fatty acid composition of PC was most readily differentiated from PE in that it contained higher levels of palmitic and myristic acids, with correspondingly lower levels of stearic acid and its unsaturated analogs. Palmitic acid, the principal fatty acid of PC, comprised about 37% of the total fatty acids. The amount of fatty acids exhibiting some degree of unsaturation represented about 41% of the total fatty acids.

The distribution pattern of fatty acids in PC was somewhat similar to that in PE, except that myristic acid was reversed in its positional distribution. As in PE, the 1-position of PC was occupied predominantly by saturated fatty acids, whereas the 2-position tended to be occupied by unsaturated fatty acids. Palmitic acid was the main saturated fatty acid in either position in PC. Only a very small portion of stearic acid was present in the 2-position. Oleic acid was distributed in both positions in quantities of 25% and 40% in the 1- and 2-positions, respectively.

The fatty acid composition of lysophosphatides showed that the naturally occurring LPE and LPC contained, in addition to saturated fatty acids, considerable quantities of unsaturated fatty acids. A comparison of fatty acids of the 1-acylglycerophosphatides obtained by phospholipase A hydrolysis of the diacylglycerophosphatides with those of natural LPE and LPC showed that the latter lysophosphatides were more unsaturated than the former lysophosphatides. If the assumption is made that fatty acids of the natural LPE and LPC are located in the same manner as found in PE and PC, then the results suggest that both the 1- and 2-forms of positionsl isomers are present. The presence of two types of positional isomers of naturally occurring lysophosphatides was further supported by the degradation studies with phospholipase C from *B. cereus*. This enzyme has been shown to act on both the 1- and 2-forms of lysophosphatides so as to form 1- and 2-monoglycerides, respectively (19).

Separation of the isomeric monoglycerides was accomplished on boric acid-impregnated TLC plates, as shown in Figures 2 and 3. The results obtained from the fatty acid analysis of natural LPE and LPC, and TLC separation of their isomeric monoglycerides demonstrated the occurrence of both the 1- and 2-forms of monoacylgycerophosphatides.

DISCUSSION

The presence of lysophosphatides has been

reported in bovine and some mammalian milks ranging from traces up to 5% of the total phospholipids with considerable variation between species (12,13). Lysophosphatides have also been found in phospholipids from the lipid extracts of human blood serum (22-24), and rat and human liver (19,25). However, the origin of lysophosphatides is not clearly understood and there has been controversy regarding the natural occurrence of lysophosphatides.

Morrison (13) inferred that the small amounts of lysophosphatides found in bovine milks are breakdown products formed during the processing of the milk. However, he also noted the presence of appreciable amounts of LPE from fresh samples of human milk, which were solvent-extracted immediately upon reception. On the other hand, from a number of control experiments carried out, Misra (24) demonstrated that LPE is not produced during extraction of chromatographic purification of serum lipids, but is a naturally occurring constituent of phosphoglyceride mixtures. The transesterification of the PC and cholesterol of high density lipoproteins by the enzyme lecithin-cholesterol acyltransferase is probably the principal source of LPC in the plasma, although the activity of tissue and plasma phospholipases undoubtedly contributes to the physiological occurrence of lysophosphatides (26). Since phospholipase activity in animal tissues has been shown to catalyze the hydrolysis of both fatty acid ester linkages of phospholipids (27), it seems reasonable to assume that the occurrence of lysophosphatides in milk phospholipids could have originated in a similar fashion.

The positional distribution of fatty acids in PE and PC determined by phospholipase A hydrolysis showed that the saturated fatty acids were located mainly at the 1-position, whereas unsaturated fatty acids tended to be preferentially esterified at the 2-position as previously noted (10, 28). The fatty acid analyses of natural LPE and LPC further revealed that these lysophosphatides contained saturated fatty acids as well as considerable quantities of unsaturated fatty acids. The degree of unsaturation of the natural lysophosphatides was much higher than that of the 1-lysophosphatides prepared by phospholipase A hydrolysis of PE and PC.

If one assumes that the fatty acids are located in the natural LPE and LPC in the same manner as found in the corresponding PE and PC, the fatty acid composition indicates that the natural lysophosphatides are mixtures of the 1- and 2-forms of positional isomers.

The possible existence of both the 1- and 2-forms of LPC in liver, lung, and spleen of rat has been reported (27). Van den Bosch and van Deenen (19) investigated in detail the nature of lysolecithins produced by rat liver with the aid of chemically synthesized lecithins containing a labeled fatty acid in a defined position, and confirmed the formation of two isomeric lysolecithins, 1-acyl-*sn*-glycero-3-phosphorylcholine and 2-acyl-*sn*-glycero-3-phosphorylcholine. The degradation of naturally occurring LPE and LPC by phospholipase C from *B. cereus,* which acts on both the 1- and 2-forms of lysophosphatides producing 1- and 2-monoglycerides, respectively (29), and subsequent separation of isomeric monoglycerides on TLC, provides further evidence for the occurrence of structurally isomeric lysophosphatides. Further studies of isomeric lysophosphatides should include a quantitative determination of the ratio of both 1- and 2-forms of these positional isomers. However, the possible migration of the acyl moiety in the 2-position to the 1-position during the isolation process, such as the column and TLC, may complicate the exact quantitations of both isomers.

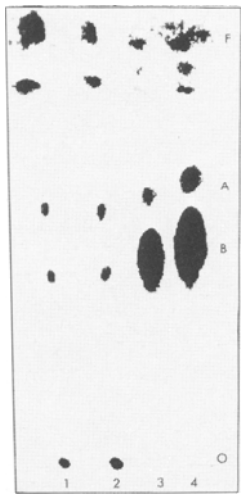

FIG. 3. Separation of isomeric monoglycerides on a boric acid-impregnated Silica Gel G TLC plate (250 μ thickness). 1. Monoglycerides obtained from natural LPE by phospholipase C hydrolysis. 2. Monoglycerides obtained from natural LPC as 1. 3 and 4. Standard mixture of 1-monopalmitin and 2-monostearin. The abbreviations and solvent system are the same as Figure 2.

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Phytyl Esters in a Marine Dinoflagellate

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ABSTRACT

The first naturally occurring phytyl esters from a marine alga were isolated from a dinoflagellate, *Peridinium foliaceum* (Stein) Biecheler, cultured photosynthetically and harvested at stationary phase of growth. At this stage, phytyl esters were the major wax esters present, constituting 5% of the total lipid, and may be present largely in the "eyespot" structure. The fatty acids esterified to the phytol were predominantly polyunsaturated: 16:3 n-3 (15%), 18:5 n-3 (6%), 20:5 n-3 (36%), and 22:6 n-3 (17%).

INTRODUCTION

Wax esters are not common constituents of algae (1,2). In fact, typical wax esters (i.e., fatty acids esterified with a homologous series of long, straight chain alcohols) have been detected in only two algal species: *Euglena gracilis* (3) and *Chroomonas salina* (4). The wax esters are especially prominent when these species are grown heterotrophically.

In contrast, many species of land plants do synthesize wax esters as cuticle constituents. Recently wax esters composed of fatty acids esterified to the terpenoid alcohol phytol have been isolated from senescent leaves of *Acer platanoides* (5), from leaves of *Fatsia japonica* (6), and from a grass, *Phleum pratense* (particularly in frost-damaged specimens of the latter) (7). Phytol, as well as phytenic acid, are the major components of wax esters in several moss species that have been examined (8).

Our investigation of the lipids of *Peridinium foliaceum*, a dinoflagellate which apparently harbors a chrysophyte-like endosymbiont (9,10), revealed a wax ester fraction. Gas liquid chromatography (GLC) and mass spectral analyses indicated that the *P. foliaceum* wax esters, unlike those of other aquatic plants which have been studied (3,4), are phytyl esters, mainly of long chain polyunsaturated fatty acids.

MATERIALS AND METHODS

Cells of *P. foliaceum* (Stein) Biecheler (IUCC No. 1688) were grown in GPC medium with 24 hr illumination (10) and harvested in stationary phase growth (14-21-days-old). Pigments were extracted as described previously (10); chlorophyll 'a' was determined spectrophotometrically (11). Lipids were extracted from 10 g wet weight of cells by sonication at medium intensities (pulsed at 30 sec intervals) in a small volume of chloroform-methanol (2:1 by vol) cooled in ice (12), The extract was diluted to 60 ml with chloroform, shaken, 40 ml of 1:1 chloroform-methanol were added, and the phases allowed to equilibrate. The hypophasic (lower) layer was then drawn off, and the solvent removed in a rotary evaporator. The residue in 10 ml of 19:1 chloroform-methanol was passed through columns of anhydrous Na_2SO_4 (to remove water) and Sephadex 25 (to eliminate amino acids, sugars, and other polar residues) and dried under nitrogen. The crude lipid was separated on a silicic acid column, eluting the neutral lipids with chloroform and the polar lipids with methanol.

Wax Ester Separation and Identification

A crude wax ester fraction was isolated from the total neutral lipids by column chromatography (13) on Unisil (Clarkson Chemical Co., Inc., Williamsport, PA), monitoring by thin layer chromatography (TLC). Wax esters were eluted with 5 column volumes (5 V) of 3% and 3 V of 10% diethyl ether in petroleum ether (bp 60-70 C, redistilled). The total wax esters were further fractionated by preparative TLC in either of two systems. System No. 1 used Silica Gel G plates ('Uniplates,' Analtech, Inc., Newark, DE) prewashed overnight with chloroform and developed with benzene-petroleum ether (30:70, v/v). Standards of authentic wax ester (hexadecyl palmitate, J. Hinton, Valparaiso, FL) and triglyceride (avocado oil) were run simultaneously. Rhodamine 6G spray was used to visualize the following lipid bands: hydrocarbons (near the solvent front), wax esters of R_f 0.56, and triglycerides at the origin. The second TLC system (No. 2) employed MgO plates (14) (J.T. Baker No. 2477, "suitable for chromatography," sieved through 100 mesh, no binder added) developed with diethyl ether-petroleum ether-ethyl acetate (35:65:1, v/v/v) (15). In this system, authentic wax esters had an R_f of 0.66 and sterol esters R_f 0.5, with a clean separation of the two bands. The various zones were scraped off with a razor blade and eluted with benzene, then 10% methanol in benzene (or 20% diethyl ether-benzene). The

adsorbent was centrifuged out, and the 'wax ester' or 'sterol ester' fractions were transmethylated in screw-capped vials fitted with Teflon liners in 0.5 N sodium methylate in methanol (Applied Science Labs., State College, PA) diluted with benzene 3:1 (v/v) by heating at 80 C for 3 min; cf. Glass (16) and Luddy et al. (17).

The crude methyl esters were either analyzed directly by GLC or were purified by preparative TLC in System No. 1, in which they had an R_f 0.21-0.25, the free alcohol/sterol moieties remaining at the origin. The GLC analysis of all methyl esters was carried out in a Varian Model 2100 instrument using glass columns of 0.2 mm ID. A 1.8 m 3% OV-101 on 100/120 Gas-Chrom Q (Applied Science Labs.) column was temperature programmed from 130-260 C (at 4 or 6 C/min) or used isothermally at 220 C. A 0.9 m 15% DEGS on 100/120 Gas-Chrom P (HI-EFF 1BP, Applied Science Labs.) was used isothermally at 160 and 190 C. The free alcohol/sterol fraction was also examined by GLC using the same conditions and columns as for the methyl esters. In addition, both the free alcohols and their trimethylsilyl ethers (TMS derivatives) were subjected to GC-mass spectrometry in a 1.5 m x 3 mm OD OV-1 column at 220 C in a Varian Model 1400 gas chromatograph coupled to a Finnigan Model 3000 quadrapole mass spectrometer. The TMS derivatives were prepared with Tri-Sil (Pierce Chemical Co., Rockford, IL); authentic phytyl TMS ether was similarly made from phytol ("triple distilled," Matheson, Coleman & Bell, Norwood, OH).

Oil Globule Lipids

Lipids from the "eyespot" or "oil globule" fraction were obtained from stationary phase cell ruptured by passage through a Yeda pressure cell at 4,200 psi nitrogen; the bright-orange globules were isolated by centrifugation in a discontinuous sucrose gradient, as described previously (10), and their purity confirmed by electron microscopy. The lipids were extracted from this subcellular fraction by the methods described for whole cells, omitting the sonication step.

RESULTS

Wax esters accounted for ca. 6% of the total lipids in *P. foliaceum;* 8.1 mg were obtained from the 129 mg total lipid extracted from 1.52 g dry wt of whole cells. This amounts to 4.5×10^{-8} mg wax ester/cell; the total chlorophyll 'a' per cell was 1.9×10^{-8} mg in this culture.

Identification of Phytol

GLC of either the crude *P. foliaceum* lipid or its total "wax ester" fraction, using conditions that gave excellent patterns for known C_{28}-C_{36} wax esters, produced only an unsteady base line. This is in agreement with the experience of Gellerman et al. (7) that phytyl esters apparently decompose when subjected to GLC.

Following transmethylation of the purified wax ester (R_f 0.56 on Silica Gel G plates) and GLC of the crude methyl esters (i.e., an aliquot of the water-washed reaction mixture was injected onto the GLC column), the major component (47%) observed had a retention time only slightly different from both 18:3 n-3 and 18:3 n-6 fatty acid methyl esters. The major peak could be partially resolved from 18:3 n-3 (methyl α-linolenate). Its equivalent chain length (ECL) was 17.78 on OV-101, 19.95 on DEGS, both at 160 C.

By preparative TLC on Silica Gel G, the methyl esters were separated from the alcohols/sterols. The resulting purified methyl esters contained a small 18:3 n-3 peak, while the ECL 17.78 (on OV-101) peak was found in the free alcohol fraction as the major component, together with a much smaller peak of ECL 19.8 and six small peaks in the sterol region, the largest probably cholesterol. This free alcohol/sterol fraction was examined by GC-mass spectrometry both free and as the TMS derivatives. The latter gave a small mass peak at m/e 368, the m/e 353 (M - 15; loss of CH_3) and m/e 278 (M - 90; loss of $[(CH_3)_3SIOH]$) peaks expected for TMS ethers (18), and major peaks at m/e 143 $[(CH_3)C{=}CHCH_2OSi(CH_3)_3]^+$, m/e 74, 72, and a small peak at m/e 213 (the isoprene homolog of m/e 143) and the ion derived from it by loss of trimethylsilanol, m/e 123. These facts lead to the empirical formula of $C_{20}H_{39}O$ for the free alcohol and its identification as phytol, 3,7,11,15-tetramethyl-2-hexadecen-1-ol. The TMS derivative of authentic phytol showed a mass spectrum indistinguishable from the *P. foliaceum* derivative. Note that at no time during the transesterification or TMS-derivative formation was free phytol exposed to acid; thus allylic rearrangement was precluded, as evidenced by the single GLC peak observed. The *P. foliaceum* wax esters, then, were largely fatty acid esters of phytol.

The total wax ester fraction isolated by column chromatography was separated into two components by preparative TLC on MgO plates (System No. 2). The major component had R_f 0.66, identical to that of hexadecyl palmitate, and the slower-moving zone R_f 0.5 (approxi-

mate), close to that of cholesteryl laurate. When the R_f 0.66 material was transmethylated as described above, the only alcohol moiety detected was phytol, identified from its TLC properties and retention times in GLC. We, therefore, regard the R_f 0.66 material as pure phytyl esters; the composition of its purified methyl esters is given in Table I. Thus, the principal wax esters of *P. foliaceum* were phytyl esters of fatty acids, mainly polyunsaturated 16:3, 18:5, 20:5, and 22:6.

Separation of wax ester types on MgO reflects the three dimensional structures of the molecules (14), e.g., the resolution of fatty acid esters of n-alkanols from those of 3-hydroxy-Δ^5-sterols. For the *P. foliaceum* wax esters, the observed difference in fatty acid composition between the R_f 0.66 and R_f 0.5 components is significant (higher 16:0 and lower 20:5 and 22:6 contents) but cannot account for the observed complete resolution of the two wax ester types, even recognizing that the basic transmethylation used would have made the detection of minor amounts of phytenic acid uncertain (7). Note that the R_f 0.5 wax esters moved with sterol esters on MgO, and Gellerman et al. (7) separated wax esters containing major proportions of phytyl and phytenoyl moieties from sterol esters on a column of MgO. Therefore, the principal difference between the *P. foliaceum* R_f 0.66 and R_f 0.5 wax esters must be in their respective alcohol moieties. To date, we have not identified the alcohols/sterols present in the more polar wax esters; in fact, no peaks assignable to alcohols or sterols were identified on GLC of the transmethylation reaction mixture from the R_f 0.5 material. We did not specifically look for phytenic or phytanic acids. Despite the absence of authentic standards and the use of basic conditions which would have lead to the formation of isomers of phytenic acid during transmethylation (7), we believe that only small amounts, if any, of these two acids occur in *P. foliaceum* phytyl esters.

Identification of 18:5 Fatty Acid

The identification of the 18:5 fatty acid component in Table I is based on the observed equivalent chain lengths for this homolog on both nonpolar (1.8 m OV-101 at 160 and 180 C, ECL 17.24-17.34) and polar columns (0.9 M DEGS at 160 C, ECL 21.96; and 1.8 m SP-2340 at 180 C, ECL 21.63-21.79), and the literature reports that 18:5 occurs in many dinoflagellates (19). Our observed ECL values are in agreement with those reported by Joseph (19) and Ackman's group (20,21), of 17.12-17.21 on Apiezon L and 17.41 on SE-30 columns, and 20.13-20.21 for Silar 5CP and 20.52 on BDS, both phases somewhat less polar than our SP-2340 and DEGS. On the DEGS column at 190 C there is overlap with authentic 20:3 n-3 of ECL 22.06-22.17. However, these two esters are easily distinguished on OV-101.

TABLE I

Fatty Acid Composition of *P. foliaceum* Phytyl Esters

Fatty acid	ECL DEGS, 190 C	Wt % Methyl esters
12:0	(12.00)[a]	0.4
14:0	14.00	1.9
16:0	16.00	2.4
16:1	16.58	8.1
16:2[b]	17.51	2.5
16:3 n-3	18.16	14.8
18:0	(18.00)	0.5
18:1	18.49	2.9
18:2	19.25	0.7
18:4[b]	20.58	0.8
18:5 n-3	22.20	5.8
20:1[c]	(20.44)	3.9
20:2	21.18	0.6
20:4	22.64	tr
20:5 n-3	23.19	35.9
22:5 n-3	25.14	1.5
22:6 n-3	25.63	17.3

[a]ECL values in parentheses derived from standards; on DEGS at 190 C these methyl esters were not resolved from other components of this sample.

[b]Tentative identification.

[c]May also include 18:3 n-3, ECL 20.24.

Occurrence in Eyespot

TLC of lipid extracted from purified carotenoid-rich eyespots of *P. foliaceum* indicated the presence of a wax ester/sterol ester fraction in addition to hydrocarbons, triglycerides, and polar lipids. No free sterol was detected after saponification, and GLC examination indicated the probable presence of phytol. The lipids of the extraplastidic eyespot lipids had a higher content of wax esters than did the whole cell lipids, but since no other subcellular fractions were individually examined and because of the possible contamination of the eyespot preparation with other "osmiophilic globules" derived from the chloroplasts (cf. Greenwood et al. ref. 22), we cannot say at this time that all – or even most – of the phytyl esters were present in the eyespots.

DISCUSSION

This is the first report of naturally occurring phytyl esters in a marine alga. It is noteworthy that the dinoflagellate, *P. foliaceum*, was grown photosynthetically but was not harvested until stationary phase, when the total lipid per cell

had increased threefold over log-phase cells. These senescent cells are particularly rich in neutral lipids found accumulated extraplastidically in carotenoid-rich osmiophilic globules [part of a membrane-bound "eyespot" structure (23)]. Wax esters (with straight chain alkyl groups, however) have also been detected in purified eyespots from *Euglena gracilis* (3). In this sense, waxes in eyespots of both *P. foliaceum* and *E. gracilis* may fulfill functions similar to those of copepod oil sac wax: acting as a potential energy reserve, a storage of polyunsaturated fatty acids, and a possible aid in buoyancy for the motile organism (24).

Screening of additional phytoplankton species for phytyl esters will be the subject of future work. Perhaps phytyl esters have escaped detection in previous studies (1,2) because they are only present in trace quantities in log phase cells, accumulating as the cells senesce. The identification of the alcohol/sterol moieties of the more polar wax ester fraction of *P. foliaceum* will also be a future concern.

It is well known that chlorophyllase can hydrolyze phytol from chlorophyll 'a,' as well as nonspecifically reesterify the side chain to chlorophyllide 'a.' This has been demonstrated in vitro (25). Free phytol has been reported as a natural entity occurring in some species of algae (26); this is probably an artifact of the known activity of chlorophyllase in organic solvents (25,27). Barrett and Jeffrey assayed chlorophyllase in marine algae and found high levels of activity in diatoms and chrysophytes, but low activities for dinoflagellates, cryptomonads, and red algae (27). In *P. foliaceum,* the proposed chrysophyte endosymbiont may be expected to assure high chlorophyllase activity. In this species, chlorophyll 'a' has an apparent rapid turnover in the cell; its relative concentration increases more than that of any other photosynthetic pigment when the cells are grown in dim light (Withers, unpublished observation). Perhaps phytyl esters are important in the metabolism of chlorophyll 'a' (via chlorophyllase) in *P. foliaceum.*

The fatty acid composition of the *P. foliaceum* phytyl esters differs from that found in higher plants and mosses in the predominance of the long chain polyunsaturated fatty acids in the dinoflagellate and the apparent absence of phytenic acid. The presence of 18:5 n-3 fatty acid in *P. foliaceum* phytyl esters is worth noting, since this fatty acid has been detected only in some dinoflagellates (11 species) but in no chlorophyte, chrysophyte, or bacillariophyte examined to date (19,20; Joseph, personal communication). This implies that the host dinoflagellate to which the eyespot pertains (10,23), *P. foliaceum,* contributes to the fatty acids esterified to phytol. Our use of $NaOCH_3$-catalyzed transmethylation would have converted any phytenic acid originally present into a mixture of isomers (7); since we did not identify any of these isomers, phytenic acid could have been only a minor constituent at most. Nor was phytanic acid detected. It appears that this group of isoprenoid acids is a major constituent of phytyl esters only in mosses (7,8). In other higher plants, the phytol is esterified to the more usual fatty acids, principally 16:0, 18:2, and 18:3 n-3 (5-7). Although the principal fatty acid of the phytyl esters of four species of mosses was phytenic acid, in the corresponding sterol ester fractions, 80% of the acyl groups were highly unsaturated C_{20} acids (8). For *P. foliaceum,* 20:5 and 22:6 fatty acids occur in higher proportions in the phytyl esters than in the total fatty acids or the neutral lipids as a whole.

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Triglyceride Composition of Bovine Milk Fat with Elevated Levels of Linoleic Acid

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ABSTRACT

The effect of increasing the linoleic acid (18:2) content of milk fat on the composition and structure of the triglycerides (TG) was investigated. Protected sunflower seed supplement was added to the diet of a cow grazing on pasture, and the structure and composition of the milk fat compared with the milk fat from its monozygous twin which had been fed a control diet. The relative proportions of TG fractions of high, medium, and low molecular weight in the milk fat with elevated levels of 18:2 (15.5% 18:2) were 43.0, 19.5, and 37.5 moles %, respectively, compared with 36.1, 19.7, and 44.2 moles %, respectively, in the milk fat from the cow fed the control diet. Separation of these three TG fractions of each milk fat into TG classes with different levels of unsaturation showed that the milk fat with elevated levels of 18:2 contained higher proportions of diene, triene, and tetraene TG and correspondingly lower proportions of saturated and, to a lesser extent, monoene TG. The saturated and monoene TG from the two milk fats had similar fatty acid compositions. However, the diene TG of the 18:2-rich milk fat included high proportions of the combination of 18:2 with two saturated fatty acids (FA) which are minor constituents of normal milk fats. Likewise, the triene TG reflected the presence of 18:2 in combination with 18:1 and a saturated FA.

INTRODUCTION

The metabolism of lipids in the rumen ensures that the fatty acid composition of bovine and other ruminant tissue and milk fats is not markedly affected by the nature of the dietary lipids (1). Consequently, it has not been possible to examine the effects of different dietary fatty acids upon triglyceride structures in ruminant fats by conventional feeding as has been done with nonruminants (2,3). However, the development of techniques for encapsulating oils and fats in formaldehyde-protein (4,5), thus protecting the fat from microbial metabolism in the rumen, enables the fatty acid composition of ruminant fats to be varied beyond the normal narrow limits. For example, the absorption of linoleic acid (18:2) following the feeding of encapsulated supplements containing 18:2-rich oils has led to tissue and milk fats with considerably elevated linoleic acid levels (6).

This investigation examines the effect of the availability of linoleic acid for milk fat synthesis on the composition and structure of the triglycerides of bovine milk fat using the milk fat from a pair of monozygous twin cows, one of which was fed formaldehyde-treated sunflower seed. The wide range of molecular weights of the constituent fatty acids in bovine milk fat gives rise to special problems in analysis of triglycerides and necessitates a preliminary fractionation of the triglycerides to give fractions of high, medium, and low molecular weight (7,8).

EXPERIMENTAL PROCEDURES

Milk from a pair of monozygous twin Jersey/Friesian cows grazing on a mixed ryegrass-clover pasture was collected and the fat analyzed over a period of 19 days. During the first week each cow was fed 1.5 kg dried grass at the morning and evening milking. Cow 1 was retained on this diet of fresh pasture and dried grass throughout the experimental period, and after the first week cow 2 was fed twice daily a supplement of formaldehyde-treated sunflower seed (Alta Lipids [N.Z] Ltd.) mixed with the dried grass. The 18:2-rich supplement contained 41.4% lipid which contained on a molar basis 0.1% 14:0, 8.1% 16:0, 4.9% 18:0, 18.8% 18:1, and 68.1% 18:2. The amounts of protected sunflower seed supplement were increased from 0.5 to 3.0 kg/day over a 4 day period and then maintained at 3.0 kg/day for a further 8 days. The milk used for the study of triglyceride (TG) composition was collected from each cow on the final day of the experiment.

The TG were chromatographed on silicic acid columns with an eluting solvent of hexane in which the proportion of diethyl ether was

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linearly increased from 0 to 9% over 1500 ml (8), and the fractions obtained were recombined according to the carbon numbers obtained by gas liquid chromatography (GLC) of the TG to give fractions containing TG of high, medium, and low molecular weight. The three fractions gave characteristic R_f's when chromatographed on layers of Silica Gel G (8). The relative sizes and the compositions of the three fractions were reproducible in duplicate separations with both the milk fat containing low levels of 18:2 and the milk fat containing elevated levels of 18:2.

Analysis of these three fractions, rather than the total TG, overcame the problems associated with the analysis of a TG mixture of wide range of molecular weights as found in bovine milk fat. These problems were the poor resolution in thin layer chromatography (TLC) of TG of different degrees of unsaturation and of the 1,2(2,3)-diglycerides and 1,3-diglycerides formed in stereospecific analysis, and the differing rates of hydrolysis of pancreatic lipase of low and high mol wt TG. In addition, the smaller number of TG species in the three fractions compared with the total milk fat gives a clearer picture of the important TG species in the milk fat (8).

The TG fractions of high, medium, and low mol wt were further resolved into TG classes of differing levels of unsaturation by TLC on Silica Gel G (Merck) impregnated with 20% (w/v) silver nitrate, and developed in chloroform containing between 0.6 and 1.0% methanol, depending on the degree of unsaturation of the TG being separated (9). The lipid bands were detected by spraying with 2',7'-dichlorofluorescein and, after identification using standard TG, each band was eluted from the layer by the addition of 0.5 ml 1% NaCl in methanol:water (9:1, v/v) and three extractions with 5 ml diethyl ether:methanol (9:1, v/v). The relative amounts of each TG band were determined by adding methyl heptadecanoate as an internal standard before GLC of the component fatty acid (FA) methyl esters.

The three TG fractions of the milk fat containing normal levels of 18:2 were separated into seven bands, of which one contained saturated TG, two contained monoene TG, two contained diene TG, and two contained triene TG. The two diene bands were combined for FA and TG analysis as were the two triene bands. The three TG fractions of the milk fat containing elevated levels of 18:2 were generally resolved into up to ten bands of which one contained saturated TG, two contained monoene TG, two contained diene TG, two contained triene TG. The remaining three bands were not completely resolved, but contained at least four double bonds per TG. Each of the diene, triene, and polyene bands were combined for subsequent analysis.

The FA composition of the TG was determined by GLC as methyl esters after transesterification (10). Chromatographic separations were carried out using a glass column (1.83 m x 3.0 mm ID) packed with 13% DEGS (Analabs, North Haven, CT) on Chromosorb W (60-80 mesh) (Varian-Aerograph, Palo Alto, CA) fitted in a Packard Gas Chromatograph. Columns were at 50 C when the sample was injected, and then the temperature was increased at 4 C/min to a final holding temperature of 170 C. The N_2 flow rate was 40 ml/min. The detector was operated at 240 C using H_2 and air flows of 25 and 200 ml/min, respectively. The injector was held at 200 C.

Quantitative GLC of TG was carried out using a glass column (0.58 m x 2.5 mm ID) packed with 3% JXR on Gas Chrom Q (100-120 mesh) (Applied Science Laboratories, State College, PA) using a Varian-Aerograph Series 1520 Gas Chromatograph as previously described (8). The GLC was temperature programmed from 220 C to 350 C at 4 C/min with a N_2 flow rate of 150 ml/min. The detector was operated at 350 C with H_2 and air flows of 15 and 300 ml/min, respectively. The injector temperature was 325 C.

RESULTS AND DISCUSSION

Fatty Acid and Triglyceride Composition of Milk Fats

The composition of milk fat from the pair of monozygous twin cows was very similar both with respect to fatty acids (Table I) and triglyceride composition (Table II) during the preliminary feeding period. The proportion of 18:2 in the milk fat increased steadily during the 8 days following commencement of feeding the 18:2-rich supplement and thereafter remained almost constant at about 16 mole % 18:2 for the remaining 4 days of the experiment. Throughout the feeding period, 4:0, 6:0, 8:0, 18:0, and 18:1 remained relatively constant whereas the proportions of 10:0, 12:0, 14:0, and 16:0 decreased in the cow receiving the encapsulated supplement. These trends were similar to those reported in earlier studies (11-13). Concurrently, the yield of fat in the milk increased from 560 to 690 g per day with the feeding of the oilseed supplement compared with an increase from 500 to 600 g per day obtained by Pan et al. (14).

The samples selected for comparison of TG structure were collected 4 days after the optimum level of 18:2 in the milk fat was

TABLE I

Fatty Acid Composition of Milk Fat and Milk Fat Fractions Obtained from a Pair of Monozygous Twin Cows Fed a Nonsupplemented Diet (Cow 1) and Fed a Diet Supplemented with Encapsulated Sunflower Seed (Cow 2)

	Fatty acid composition (mole %)											
	Milk fat[a]											
	Cow 1		Cow 2		Milk fat[b]		High molecular weight		Medium molecular weight		Low molecular weight	
Fatty acid	Mean	Range	Mean	Range	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2
4:0	11.2	(10.5-11.8)	11.1	(10.1-11.5)	11.9	10.4	-[c]	-	5.3	8.2	25.0	24.3
6:0	5.2	(5.0- 5.3)	5.1	(4.6- 5.7)	5.3	5.4	0.4	1.1	10.4	12.3	6.2	5.9
8:0	2.2	(1.9- 2.5)	2.2	(1.9- 2.5)	2.3	2.1	1.1	1.5	4.3	3.8	2.3	2.3
10:0	3.6	(3.1- 4.2)	3.7	(3.2- 4.4)	4.1	3.2	3.8	3.7	5.9	4.5	3.6	3.7
10:1	0.2	(0.2- 0.3)	0.2	(0.1- 0.3)	0.3	0.2	-	-	0.5	0.3	0.5	0.3
12:0	3.6	(3.1- 4.0)	3.7	(3.3- 4.0)	3.5	2.6	4.0	3.2	4.5	2.6	3.8	3.4
14:0	9.7	(8.7-10.4)	9.6	(8.9-10.1)	11.9	7.7	13.3	8.7	11.8	7.3	11.5	9.1
14:1	0.9	(0.8- 0.9)	0.8	(0.7- 0.9)	1.0	0.5	0.7	0.6	1.4	0.4	0.9	0.6
15:0	0.9	(0.8- 1.1)	0.9	(0.8- 1.0)	1.0	0.7	1.3	0.7	1.0	0.6	0.9	0.6
16:0	22.0	(21.2-23.0)	20.5	(19.6-22.0)	22.1	14.1	27.3	16.1	23.6	14.0	20.2	13.1
16:1	1.9	(1.5- 2.6)	1.8	(1.7- 1.9)	2.0	1.1	1.9	1.4	2.1	0.9	1.4	1.0
17:0	0.7	(0.6- 0.9)	0.6	(0.4- 0.8)	0.9	0.5	0.9	0.7	0.8	0.6	0.4	0.2
18:0	12.7	(12.2-13.7)	14.0	(13.7-14.3)	11.9	14.1	16.4	18.5	11.1	15.3	7.6	8.0
18:1	21.0	(20.8-21.3)	22.2	(20.8-23.7)	18.6	20.6	26.5	26.5	15.3	15.9	12.6	13.2
18:2	1.8	(1.5- 2.0)	1.6	(1.1- 2.0)	1.8	15.5	1.7	16.1	1.0	11.6	1.2	13.5
18:3	1.3	(1.0- 1.9)	1.0	(0.8- 1.2)	0.8	0.7	0.7	1.1	0.8	0.7	1.2	0.8
20:?	1.0	(0.8- 1.2)	0.8	(0.6- 0.9)	0.5	0.7	-	-	0.2	1.0	0.8	-

[a]At the beginning of the trial period (day 5 to day 7).

[b]At the end of the trial period (day 19).

[c](-) = Not detected.

TABLE II

Triglyceride (TG) Composition of Milk Fat and Milk Fat Fractions Obtained from a Pair of Monozygous Twin Cows Fed a Nonsupplemented (Cow 1) and Fed a Diet Supplemented with Encapsulated Sunflower Seed (Cow 2)

	Triglyceride composition (mole %)											
	Milk fat[a]											
	Cow 1		Cow 2		Milk fat[b]		High molecular weight		Medium molecular weight		Low molecular weight	
TG[c,d]	Mean	Range	Mean	Range	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2	Cow 1	Cow 2
26	-[e]	-	-	-	-	0.1	-	-	-	-	0.2	0.2
28	0.2	(0.2- 0.2)	0.6	(0.5- 0.7)	0.7	0.6	-	-	-	-	1.3	1.9
30	0.8	(0.7- 1.0)	1.2	(1.2- 1.3)	1.4	1.1	-	-	-	-	3.4	3.9
32	2.0	(1.6- 2.3)	2.6	(2.5- 2.7)	3.0	2.1	-	-	-	0.1	8.1	7.2
34	4.7	(4.4- 4.9)	5.6	(5.3- 5.8)	6.7	3.6	-	-	1.1	1.6	18.8	10.9
36	10.4	(10.0-10.9)	11.0	(10.7-11.3)	12.3	7.5	-	-	7.7	8.3	28.0	18.3
38	15.3	(14.5-15.8)	14.7	(14.5-14.8)	14.5	11.7	-	0.9	21.6	20.8	24.9	22.9
40	13.8	(12.9-14.3)	12.9	(12.7-13.1)	12.0	16.9	0.8	1.9	24.8	28.8	13.3	30.5
42	8.0	(7.9- 8.1)	7.8	(7.7- 8.0)	7.9	9.0	3.8	4.5	19.5	19.7	1.8	4.1
44	5.8	(5.6- 6.1)	6.1	(6.0- 6.1)	6.5	5.5	7.8	6.2	10.4	7.4	0.2	0.1
46	5.7	(5.5- 5.9)	5.8	(5.7- 5.9)	6.2	5.6	11.8	8.3	6.1	4.9	-	-
48	6.5	(6.3- 6.8)	6.5	(6.3- 6.8)	6.9	5.1	17.1	9.6	3.5	1.8	-	-
50	9.6	(9.1-10.2)	9.1	(8.9- 9.5)	8.6	7.8	25.9	17.0	2.9	1.9	-	-
52	10.5	(9.9-11.2)	9.6	(9.4-10.0)	7.7	9.5	22.1	22.3	1.8	1.6	-	-
54	6.7	(6.2- 7.5)	6.4	(6.2- 6.5)	5.6	13.9	10.7	29.4	0.6	3.1	-	-

[a]At the beginning of the trial period (day 5 to day 7).

[b]At the end of the trial period (day 19).

[c]Only triglycerides of even carbon number included.

[d]Carbon number of triglycerides.

[e](-) = Not detected.

TABLE III

Proportions of Triglyceride (TG) Fractions of High, Medium, and Low Molecular Weight, and Triglyceride Classes of Differing Levels of Unsaturation within each Fraction, in Control and 18:2-Rich Milk Fat

	Proportions of the fractions and classes			
	Control		18:2-Rich	
	%F[a]	%M[b]	%F[a]	%M[b]
TG fraction of high mol wt	100.0	36.1	100.0	43.0
Saturated TG	29.5	10.6	20.0	8.6
trans-Monoene TG	9.9	3.6	3.6	1.6
cis-Monoene TG	34.1	12.3	21.1	9.1
Diene TG	17.5	6.3	27.5	11.8
Triene TG	8.9	3.2	15.6	6.7
Polyene TG	-	-	12.1	5.2
TG fraction of medium mol wt	100.0	19.7	100.0	19.5
Saturated TG	46.9	9.2	28.0	5.5
trans-Monoene TG	7.8	1.5	7.9	1.5
cis-Monoene TG	23.9	4.7	24.1	4.7
Diene TG	13.2	2.6	22.5	4.4
Triene TG	8.1	1.6	9.0	1.8
Polyene TG	-	-	8.5	1.7
TG fraction of low mol wt	100.0	44.2	100.0	37.5
Saturated TG	47.2	20.9	31.2	11.7
trans-Monoene TG	9.3	4.1	5.0	1.9
cis-Monoene TG	28.0	12.4	20.1	7.5
Diene TG	9.2	4.2	27.7	10.4
Triene TG	6.3	2.8	9.1	3.4
Polyene TG	-	-	6.9	2.6
Milk fat		100.0		100.0
Saturated TG		40.7		25.8
trans-Monoene TG		9.2		5.0
cis-Monoene TG		29.4		21.3
Diene TG		13.1		26.6
Triene TG		7.6		11.9
Polyene TG		-		9.5

[a]Molar percentage in triglyceride fraction.

[b]Molar percentage in milk fat.

attained. This milk fat from the cow fed the protected supplement (18:2-rich milk fat) contained 15.5% 18:2 compared with 1.8% 18:2 in the milk fat obtained on the same day from the cow fed the nonsupplemented diet (control milk fat) (Table I). The composition of the milk fat of the cow not receiving the encapsulated supplement was similar at the beginning and end of the feeding period (Tables I and II). The altered FA composition resulting from feeding encapsulated sunflower seed resulted in higher levels of triglycerides with 40, 52, and 54 acyl carbons (Table II) and corresponding decreases in the proportions of triglycerides with 34, 36, and 38 acyl carbons. In addition, the proportions of triglycerides with 44 to 50 acyl carbons were slightly lower in the 18:2-rich milk fat. In contrast, Edmondson et al. (15) observed that milk fat containing 30% 18:2 had a higher proportion of C_{54} TG but a normal level of C_{40} TG (12%) as compared with normal milk fat.

Proportions and Compositions of the Triglyceride Fractions of Differing Molecular Weight

The fractionation of the triglycerides of bovine milk fat into three subfractions, according to molecular weight, on silicic acid is a reproducible procedure as judged by this and earlier work (7,8). The size of the three subfractions of high, medium, and low mol wt obtained by column chromatography of the control milk fat were 36.1, 19.7, and 44.2%, respectively, in the present study (Table III) and in the ranges 39-41, 17-18, and 42-44% in the work of Taylor and Hawke (8). The FA and TG compositions of the subfractions were also similar to those of the earlier study (8).

A similar fractionation of milk fat containing 15.5% 18:2 demonstrated the presence of higher proportions of the high mol wt fraction and lower proportions of the low mol wt fraction than in the corresponding control milk fat, with the proportions of the high, medium,

and low mol wt fractions being 43.0, 19.5, and 37.5%, respectively (Table III). The lower and higher proportions of the low and high mol wt fractions, respectively, in the 18:2-rich milk fat as compared with the control milk fat were consistent with the triglyceride compositions of the two milk fats (Table II).

In the high mol wt fraction of the 18:2-rich milk fat, there was 29.4% C_{54} TG compared with 10.7% in the same fraction of the control milk fat. This change was accompanied by lower proportions of TG with 46, 48, and 50 acyl carbons, but there was little change in the level of the C_{52} TG. The proportions of 18:0 and 18:1 were similar in the two milk fat fractions, but the high mol wt fraction of the 18:2-rich milk fat contained higher proportions of 18:2 and correspondingly lower proportions of 14:0 and 16:0.

The TG compositions of the medium mol wt fractions from the two milk fats were similar, with the most abundant TG species being C_{38}, C_{40}, and C_{42}. Again, the increase in the proportion of 18:2 resulted in a decrease in the proportions of 14:0 and 16:0. The proportions of 18:0 tended to be higher in the 18:2-rich milk fat.

The predominant effect of the increased 18:2 content on the FA composition of the low mol wt fraction was to lower the proportion of 16:0, leaving the proportion of the other major FA, i.e., 4:0 and 18:1, relatively unchanged. As a consequence, there were consideraly higher proportions of TG with 40 acyl carbons (30.5% compared to 13.3%) and lower proportions of TG with 34 and 36 acyl carbons in the low mol wt fraction of the 18:2-rich milk fat.

Proportions and Compositions of the Triglyceride Classes of Differing Levels of Unsaturation

The proportions of TG classes, of differing levels of unsaturation obtained in each TG fraction of low and medium mol wt, by Breckenridge and Kuksis (7) and Taylor and Hawke (8), were similar to those in the control milk fat. On the other hand, the TG fractions of high mol wt of Canadian milk fats (16,17) have been shown to have lower levels of saturated TG and higher levels of triene and polyene TG than New Zealand milk fats (8). Analysis of the proportions of TG of differing levels of unsaturation in the control and 18:2-rich milk fats, following Ag^+/TLC fractionation of the TG fractions of high, medium, and low mol wt, showed decreases in the proportions of saturated and monoene TG and increases in diene, triene, and polyene TG in the 18:2-rich milk fat (Table III). These changes occurred in each of the three fractions except that the proportions of monoene TG remained the same in the medium mol wt fraction.

Although the saturated TG comprised only 20.0% of the high mol wt fraction of the 18:2-rich milk fat compared with 29.5% in the control milk fat, the saturated TG from the two milk fats had similar compositions. They consisted primarily of 14:0, 16:0, and 18:0 arranged to give TG species containing between 42 and 50 acyl carbons (Table IV). It would appear from this and earlier work (8,17) that the proportions of saturated TG of high mol wt may vary considerably, but the composition of these TG in bovine milk fat from different sources is fairly constant. Similarly, the trisaturated TG of medium and low mol wt from the two milk fats had similar compositions (Tables V and VI) despite appreciable differences in the proportions of the trisaturated components (Table III).

As observed by Breckenridge and Kuksis (7) and Taylor and Hawke (8), the major saturated TG of low mol wt consist of 4:0 in combination with any two out of 14:0, 16:0, and 18:0 to give the principal TG species C_{34} and C_{36} (Table VI). Similarly, the proportions of both *cis* and *trans* monoene TG in the high-low mol wt fractions of 18:2-rich milk fat were much lower (Table III). There were only minor changes in the proportions of the TG of different carbon number (Tables IV-VI) and these were consistent with small changes in fatty acid composition. For example, the slightly lower proportions of C_{48} and C_{50} TG and slightly higher proportions of C_{54} TG in both monoene fractions of high mol wt can be accounted for by lower proportions of 14:0 and 16:0 and high proportions of 18:0 and 18:1. TG containing 36 and 38 acyl carbons were the most abundant TG species in the *trans* and *cis*-monoene TG of low mol wt (Table VI), and the principal FA were 4:0, 14:0, 16:0, and 18:1. Major species of TG are likely to be 4:0-14:0-18:1 and 4:0-16:0-18:1, as observed by Nutter and Privett (18).

Not only were the proportions of diene TG greater in the 18:2-rich than in the control milk fat but there were major differences in the FA compositions. The high mol wt TG of 18:2-rich milk fat contained high levels of 18:1 (29.4%) and 18:2 (18.2%) suggesting a combination of 18:2 with two saturated FA, i.e., 16:0 and 18:0, or of two 18:1's with either 16:0 or 18:0 to give mainly C_{50}, C_{52}, and C_{54} TG (Table IV), while the control milk fat contained 51.1% 18:1 and only 1.7% 18:2 to give principally C_{52} TG (Table IV), e.g., 16:0-18:1-18:1, together with C_{50} TG, e.g., 14:0-18:1-18:1,

TABLE IV

Triglyceride (TG) Composition of the Triglyceride Classes of Differing Levels of Unsaturation in the High Molecular Weight Fractions of the Control and 18:2-Rich Milk Fats

	Triglyceride composition (mole %)											
	Saturated TG		*trans*-Monoene TG		*cis*-Monoene TG		Diene TG		Triene TG		Polyene TG	
TG[a,b]	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich
36	.[c]	-	-	1.3	-	-	-	-	-	-	-	-
38	-	1.3	-	4.3	-	-	-	0.1	-	-	-	-
40	2.6	9.8	-	5.7	-	0.7	-	0.1	-	-	-	-
42	14.0	20.1	0.1	4.5	0.3	4.3	0.2	1.6	0.1	-	-	-
44	19.8	18.7	3.6	4.8	4.3	8.3	2.3	4.9	0.2	1.0	-	1.0
46	21.0	15.5	9.2	7.6	10.7	12.1	4.7	8.9	1.5	3.7	-	2.0
48	19.5	13.5	21.6	12.1	22.3	16.4	6.9	11.4	5.2	4.9	-	7.0
50	14.9	13.0	35.2	22.1	33.1	24.6	22.4	20.5	16.5	15.1	-	16.9
52	6.8	7.4	25.0	24.3	23.2	22.6	41.7	26.5	28.6	29.4	-	73.0
54	1.4	0.6	5.3	13.3	6.0	11.0	21.8	25.9	48.0	45.9	-	

[a]Carbon number of triglycerides.
[b]Only triglycerides of even carbon number included.
[c](-) = Not detected.

and C_{54} TG, e.g., 18:0-18:1-18:1. The increase in the diene TG class in the 18:2-rich milk fat relative to the control milk fat (27.5% compared to 17.5%) was largely due to the presence of TG containing one 18:2 and two saturated FA. The contribution of TG containing two monoenoic FA was a little lower than in the control milk fat (12% compared to 17%). There was a threefold increase in the quantitative importance of the diene TG in the low mol wt fraction of 18:2-rich milk fat compared with the control milk fat (Table III). The more abundant diene TG in the control milk fat consisted of the TG species C_{40} (Table VI) containing the FA 4:0 and 18:1, i.e., 4:0-18:1-18:1 (7,18). Moreover, the dienes of the 18:2-rich milk fat contained only low levels of 18:1, and instead there were high levels of 18:2. The abundance of C_{38} and C_{40} TG (Table VI) suggests that the principal TG species were 4:0-16:0-18:2 and 4:0-18:0-18:2 rather than species such as 4:0-18:1-18:1 in the control milk fat.

The triene TG comprised 8.9% and 15.6% of the high mol wt fraction of the control and 18:2-rich milk fats, respectively (Table III). Almost half the triene TG of high mol wt in both milk fats contained 54 acyl carbons and more than one-quarter contained 52 acyl carbons. In the control milk fat fraction, 18:1 (46.0%) was the most abundant FA together with lesser amounts of 18:2 (15.9%). However, in the 18:2-rich milk fat, 18:1 and 18:2 were in the approximate ratio of 1:1, suggesting that 16:0-18:1-18:2 and 18:0-18:1-18:2 were the principal TG species. In the 18:2-rich milk fat, the principal triene TG of low mol wt had 40 acyl carbons and the major FA were 4:0, 18:1, and 18:2 in similar proportions. However, in the control milk fat, TG with 38 and 40 acyl carbons made up considerable proportions of the triene TG, and 4:0, 16:0, 18:1, and 18:3 were the most abundant FA.

In addition, the high mol wt fraction of the 18:2-rich milk fat contained 12:1% polyene TG. TG and FA analysis indicated that a large proportion of the TG of this fraction consisted of various combinations of 18:1 and 18:2. The polyene TG which were absent in the control milk fat comprised 8.5% of the medium mol wt fraction of the 18:2-rich milk fat with major TG being C_{46}, C_{50}, C_{52}, and C_{54} and the major FA 18:2 (46.8%), with lesser amounts of 18:1 (11.8%). Likely combinations would consist of two 18:2's with either 10:0, 14:0, 16:0, 18:0, or 18:1. The polyene TG of the 18:2-rich milk fat contained high proportions of 4:0 and 18:2 with the most abundant TG species having 40 and 42 acyl carbons to give

TABLE V

Triglyceride (TG) Composition of the Triglyceride Classes of Differing Levels of Unsaturation in the Medium Molecular Weight Fractions of the Control and 18:2-Rich Milk Fats

	Triglyceride composition (mole %)											
	Saturated TG		*trans*-Monoene TG		*cis*-Monoene TG		Diene TG		Triene TG		Polyene TG	
TG[a,b]	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich
32	0.1	2.0	-[c]	-	-	-	-	-	0.1	-	-	-
34	3.1	8.1	0.3	0.9	-	0.2	0.2	-	0.8	-	-	-
36	17.0	26.1	8.3	5.0	1.2	3.6	0.8	0.2	2.3	-	-	-
38	33.5	36.7	19.3	23.0	15.0	23.6	3.4	5.1	3.5	-	-	-
40	24.8	20.9	31.7	41.8	32.1	44.7	11.5	34.5	4.3	2.9	-	1.2
42	12.3	5.0	20.3	20.1	23.4	20.2	21.1	36.4	6.5	40.2	-	5.8
44	5.4	0.9	9.4	6.1	13.5	4.6	18.2	12.0	7.8	19.4	-	7.6
46	2.3	0.3	5.1	2.9	7.4	1.9	16.7	6.3	12.7	17.5	-	12.9
48	1.1	-	3.4	0.2	4.7	0.6	11.1	2.5	12.9	5.5	-	6.8
50	0.4	-	2.0	0.1	2.5	0.5	10.1	1.9	18.3	6.0	-	11.1
52	-	-	0.3	-	0.3	0.1	5.5	0.9	16.7	4.3	-	13.3
54	-	-	-	-	-	-	1.5	0.3	12.1	4.2	-	39.0
56	-	-	-	-	-	-	-	-	2.0	-	-	2.2

[a]Carbon number of triglycerides.

[b]Only triglycerides of even carbon number included.

[c](-) = Not detected.

TABLE VI

Triglyceride (TG) Composition of the Triglyceride Classes of Differing Levels of Unsaturation in Low Molecular Weight Fractions of the Control and 18:2-Rich Milk Fats

	Triglyceride composition (mole %)											
	Saturated TG		*trans*-Monoene TG		*cis*-Monoene TG		Diene TG		Triene TG		Polyene TG	
TG[a,b]	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich
26	-[c]	0.5	-	-	-	-	-	-	-	-	-	-
28	1.8	4.3	-	-	-	-	-	-	-	-	-	-
30	5.7	9.7	-	-	0.4	0.7	-	-	-	-	-	-
32	13.9	16.7	0.4	2.2	3.2	5.4	8.2	0.8	3.3	-	-	0.8
34	30.2	27.4	14.0	9.2	9.2	11.2	12.0	3.2	3.4	1.2	-	0.8
36	35.2	28.8	27.5	28.8	29.4	27.1	10.5	11.6	12.5	2.1	-	2.4
38	12.4	11.8	47.0	40.0	45.6	37.7	15.4	35.2	25.5	4.1	-	5.7
40	0.7	0.8	11.1	19.8	12.0	18.0	50.1	45.5	47.2	76.2	-	64.9
42	-	-	-	-	0.2	-	3.7	3.7	6.8	15.4	-	20.8
44	-	-	-	-	-	-	-	0.1	1.4	1.0	-	3.1
46	-	-	-	-	-	-	-	-	-	-	-	1.5

[a]Carbon number of triglycerides.

[b]Only triglycerides of even carbon number included.

[c](-) = Not detected.

the likely major combinations of 4:0-18:2-18:2, and lesser amounts of 6:0-18:2-18:2.

Comparison of the Triglyceride Classes of Differing Levels of Unsaturation

The TG compositions of the saturated TG of the two milk fats calculated from the analysis of the separate fractions were similar except for slightly lower proportions of C_{34} and C_{36} in the 18:2-rich milk fat (Table VII). The *trans* and *cis*-monoene TG of 18:2-rich milk fat tended to have higher proportions of C_{40} and lower proportions of C_{50} than the corresponding TG classes in the control milk fat. There were appreciable differences between the TG compositions of the diene TG of the control and 18:2-rich milk fats, with the 18:2-rich milk containing higher proportions of C_{38} and C_{40} and lower proportions of C_{50} and C_{52} (Table VII).

The triene TG of the 18:2-rich milk fat contained lower proportions of C_{38} and higher proportions of C_{40} and C_{42} than the triene TG of the control milk fat. The proportions of C_{50}, C_{52}, and C_{54} were similar.

The monoene, diene, and triene TG classes of the 18:2-rich milk fat generally contained higher proportions of the TG of lower mol wt and, in the case of the monoene and diene TG classes, lower proportions of the TG of higher mol wt suggesting an overall increase in low mol wt TG. However, when the relative contribution of these TG classes to the total milk fats was taken into account, because of the increase in size of the diene and triene TG classes and the emergence of a polyene TG class, the level of C_{50} and C_{52} TG in the total milk fats was the same, and the level of C_{54} TG in the 18:2-rich milk fat was actually higher than in the control milk fat. It would appear that the contribution of the C_{50} and C_{52} TG of the monoene and diene TG to the total milk fat was relatively unaltered by feeding high levels of 18:2, but the contribution of C_{40} TG in the monoene, diene, and triene TG to the total milk fat was much increased. The increased proportions of C_{54} in the 18:2-rich milk fat mainly arose from the presence of the polyene TG which were comprised of 47% C_{54} and contributed almost 40% of the C_{54} in the 18:2-rich milk fat. The TG composition of the milk fat calculated from the composition of the fractions was very similar to that observed by direct analysis (Tables II and VII).

It is concluded that the greatest effect of increasing the amount of 18:2 in milk fat was to reduce the proportions of saturated TG of low mol wt in the total milk fat with smaller

TABLE VII

Triglyceride (TG) Composition of the Triglyceride Classes of Differing Levels of Unsaturation in the Control and 18:2-Rich Milk Fats[a]

	Triglyceride composition (mole %)													
	Saturated TG		*trans*-Monoene TG		*cis*-Monoene TG		Diene TG		Triene TG		Polyene TG		Milk fat[d]	
TG[b,c]	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich	Control	18:2-Rich
26	-[e]	0.2	-	-	-	-	-	-	-	-	-	-	-	0.1
28	0.9	2.0	-	-	-	-	-	-	-	-	-	-	0.4	0.5
30	2.9	4.4	-	-	0.2	0.2	-	-	-	-	-	-	1.2	1.2
32	7.1	8.0	0.2	0.8	1.4	1.9	2.6	0.3	1.2	-	-	0.2	3.8	2.6
34	16.1	14.2	6.3	3.7	3.9	4.0	3.9	1.3	1.4	0.3	-	0.2	8.9	5.1
36	21.8	18.6	13.6	12.9	12.6	10.3	3.5	4.6	5.1	0.6	-	0.7	14.7	9.0
38	13.9	13.6	24.1	23.5	21.6	18.5	5.6	14.7	10.1	1.2	-	1.6	15.7	12.8
40	6.6	8.1	10.1	21.8	10.2	16.5	18.3	23.5	18.3	22.2	-	18.0	10.4	17.3
42	6.5	7.8	3.4	7.5	4.0	6.3	5.5	8.2	3.9	10.5	-	6.7	5.2	7.8
44	6.4	6.4	2.9	3.4	4.0	4.6	4.7	4.2	2.2	3.8	-	2.2	4.8	4.6
46	6.0	5.2	4.4	3.3	5.7	5.6	5.6	5.0	3.3	4.7	-	3.3	5.5	4.9
48	5.4	4.5	9.0	3.9	10.1	7.1	5.5	5.5	4.9	3.6	-	2.3	7.1	5.0
50	4.0	4.3	14.1	7.1	14.2	10.6	12.8	9.4	10.8	9.4	-	5.8	9.6	7.9
52	1.8	2.5	9.8	7.8	9.8	9.7	21.1	11.9	15.6	17.2	-	11.6	8.5	9.4
54	0.4	0.2	2.0	4.3	2.5	4.7	10.8	11.5	22.8	26.5	-	46.9	4.2	11.9
56	-	-	-	-	-	-	-	-	0.4	-	-	0.4	-	-

[a]Calculated from the TG compositions of the TG classes of the respective TG fractions.

[b]Carbon number of triglycerides.

[c]Only triglycerides of even carbon number included.

[d]Calculated from the analysis of each fraction.

[e](-) = Not detected.

reductions in the saturated TG of high molecular weight. Conversely, the greatest increase occurred in the proportions of diene TG of low and high mol wt with contributions from this TG class increasing from 4.2 to 10.4% and from 6.3 to 11.8%, respectively. A large increase also occurred in the proportions of tetraene TG of high mol wt. These trends in milk fat containing 15.5 moles % 18:2 possibly would be more marked in milk fat containing greater proportions of 18:2 (15).

Although there was a decreased contribution of saturated and monoene TG to the total TG, the composition of each of these TG classes was similar in the two milk fats indicating the presence of similar TG species. However, the data indicated that TG such as 4:0-18:1-18:1 and 16:0-18:1-18:1 contributing significantly to the TG of normal milk fat are supplemented in importance by diene, triene, and tetraene TG containing 18:2, e.g., 4:0-16:0-18:2, 4:0-18:1-18:2, and 4:0-18:2-18:2. Similar changes have been observed in the depot fat of the rat (3) with the feeding of diets high in 18:2, such as corn oil, where proportions of 16:0-18:2-18:1, 18:1-18:2-18:1, 16:0-18:2-18:2, and 18:1-18:2-18:2 increased and proportions of 16:0-18:1-16:0 and 16:0-18:1-18:1 decreased. On the other hand, in depot fats of the dog (19) containing high levels of 18:2 (14-16%) TG species such as 16:0-16:0-18:1 (without regard to positional specificities), 16:0-18:1-18:1, 18:1-18:1-18:1, and 16:0-18:1-18:2 were found to predominate; whereas, in the seed oil from Mowrah butter (*Madhuca latifolia*), containing 14.4% 18:2, 37.6% 18:1, and 47.8% saturated fatty acids, SSM (35%), SM_2 (21%), S_2D (17%), and SMD (15%) are the most abundant TG species (20).

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Positional Distribution of Fatty Acids in the Triglycerides of Bovine Milk Fat with Elevated Levels of Linoleic Acid

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ABSTRACT

A stereospecific distribution of fatty acids in bovine milk fat containing 15.5% linoleic acid has been compared with the distribution in bovine milk fat containing a normal level (1.8%) of linoleic acid. The positional distribution was obtained by the separate analysis of milk fat triglycerides of high, medium, and low molecular weight. The order of preference for linoleic acid in the high molecular weight triglycerides was position 3 > position 2 > position 1. There was an accompanying altered distribution of myristic acid and palmitic acid in favor of position 1 at the expense of position 3. However, the proportions of myristic acid and palmitic acid in position 2, relative to positions 1 and 3 were identical in the high molecular weight fractions of the two milk fats. The distribution of linoleic acid in the medium molecular weight triglycrides of linoleic-rich milk fat was position 1 = position 2 > position 3. This resulted in a change in the distribution of 18 carbon monounsaturated fatty acids in favor of position 2 at the expense of position 1, but the distribution of myristic acid and palmitic acid was virtually unaltered. The distribution of linoleic acid in the low molecular weight triglycerides was position 2 > position 1 > position 3. The amounts of myristic acid and palmitic acid in position 2 and of palmitic acid in position 1 decreased in the low molecular weight triglycerides of the milk fat containing elevated levels of linoleic acid. Changes in the distribution of fatty acids which were observed in the separate analysis of the high, medium, and low molecular weight triglycerides were not apparent when comparing the distribution in the total milk fats. For example, the distribution of myristic acid and palmitic acid appeared to be unchanged, while the distribution of 18 carbon monounsaturated fatty acids was slightly altered in favor of positions 2 and 3. Moreover, linoleic acid showed an almost equal preference for the three positions of the glycerol moiety in milk fat containing elevated levels of this fatty acid with some concentration at position 2.

INTRODUCTION

Linoleic acid shows a preference for position 2 in the great majority of plant and animal depot triglycerides (1). This structural preference was maintained when rats were fed corn oil (2) and when sheep were fed protected safflower oil (3) although appreciable 18:2 also entered position 3 (4).

The positional distribution of 18:2 in the milk fat of ruminants has been determined only in milk fats containing low levels of this fatty acid (FA) (5-8) although recently Smith et al. (9) have used the pancreatic lipase technique to examine polyunsaturated milk fat. These studies indicated that 18:2 in bovine milk fat, as in other animal fats excepting that of the pig (10), preferred position 2 of these triglycerides although the inaccuracies in determining the distribution of minor components should be recognized.

This investigation examines the stereospecific distribution of 18:2 in milk fat with elevated levels of this fatty acid and its effect on the normal stereospecific distribution of the other constituent fatty acids. In view of the presence of short chain fatty acids in the 3-position of almost 50% of the triglycerides, bovine milk fat is an interesting natural fat in which to follow these effects. The possibility has been examined that the distribution of fatty acids in the high and low molecular weight triglycerides was affected differently by the incorporation of 18:2.

EXPERIMENTAL PROCEDURES

The milk used for stereospecific analysis was obtained from a pair of monozygous twin cows fed the same basic diet. The diet of one cow was supplemented with formaldehyde-treated sunflower seed (11). The fats with normal and elevated levels of 18:2 were each separated into triglyceride fractions of high, medium, and low molecular weight (11). Stereospecific analysis of each fraction was carried out essentially by

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TABLE I

Fatty Acid Composition of the 1,2(2,3)-Diglyceride Intermediates in the Stereospecific Analysis of the Triglyceride of Milk Fat from Monozygous Twin Cows Fed Control and 18:2-rich Diets

Milk fat	Mol wt fraction		Fatty acid composition (mole %)															
			4:0	6:0	8:0	10:0	10:1	12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:1
Control	High	Exp.[a]	---	0.2	1.4	4.3	---	4.3	16.1	1.3	1.6	28.0	2.0	1.3	14.2	23.3	1.3	0.7	---
		Calc.[b]	---	0.3	1.0	3.6	---	4.4	16.1	1.0	1.3	29.7	2.0	1.8	14.1	23.5	1.6	0.7	---
18:2-rich	High	Exp.[a]	---	0.9	1.8	3.8	0.1	3.6	10.4	1.1	1.0	17.7	1.7	0.8	16.0	23.8	16.0	1.4	---
		Calc.[b]	---	0.8	1.5	3.8	---	4.0	10.7	0.5	0.7	18.0	1.5	0.6	16.4	24.5	16.1	1.0	---
Control	Medium	Exp.[a]	4.6	10.0	3.2	5.1	0.4	4.9	13.5	0.8	1.1	26.2	2.7	0.8	10.6	14.3	1.0	0.7	0.2
		Calc.[b]	4.0	8.3	4.0	6.0	0.4	5.2	14.5	1.3	1.1	25.7	2.0	0.7	10.1	14.7	1.1	0.8	0.2
18:2-rich	Medium	Exp.[a]	7.0	11.9	3.3	4.7	0.2	3.6	8.8	0.8	0.7	15.3	1.5	0.5	13.8	16.5	10.5	0.5	0.5
		Calc.[b]	6.1	9.6	4.0	5.0	0.3	3.3	9.0	0.3	0.7	15.6	1.0	0.6	14.4	16.3	12.0	0.7	0.8
Control	Low	Exp.[a]	22.5	5.9	2.4	4.5	0.7	5.2	16.1	1.0	1.0	21.0	1.3	0.1	5.6	10.3	1.0	0.7	0.3
		Calc.[b]	18.8	5.5	3.1	4.9	0.6	5.1	14.2	1.0	1.2	21.5	1.5	0.4	6.9	12.4	1.2	1.1	0.7
18:2-rich	Low	Exp.[a]	22.0	4.6	2.3	3.9	0.2	4.2	9.5	0.4	0.7	13.6	1.5	0.5	8.3	13.4	14.4	0.6	---
		Calc.[b]	18.2	5.8	3.3	5.1	0.4	4.4	10.5	0.7	0.7	13.3	1.1	0.2	7.5	13.3	14.8	0.9	---

[a]Obtained experimentally.
[b][3 x (TG) + (2-MG)] / 4.

the scheme proposed by Brockerhoff (12) and modified by Christie and Moore (13). The 1,2(2,3)-diglyceride intermediates from the fraction of high molecular weight were prepared by partial deacylation with a Grignard reagent (13) whereas those from the fractions of low and medium molecular weight were prepared by digestion with pancreatic lipase (14) as described by Taylor and Hawke (8). The 1,2-diacyl-*sn*-glycerol-1-phosphoryl phenols (2,3-PL) were prepared from the 1,2(2,3)-diglycerides (8) and purified by TLC on Silica Gel G with a solvent system of chloroform: methanol: 14M-ammonia (80:20:2, by vol). The phosphatidyl phenols were digested with phospholipiase A_2 *(Ophiophagus hannah* snake venom, Sigma Chemical Co. St. Louis, MO), then isolated and separated by TLC as previously described (8). The phosphatidyl phenols were extracted from the adsorbent with two 30 ml portions of chloroform:methanol:water (60:30:3, by vol) and two 30 ml portions of chloroform:methanol:water (30:60:3, by vol). Solvents were either evaporated under N_2, or, alternatively, the bulk of the solvent was removed on a rotary evaporator at temperatures below 35 C and the remaining solvent removed under N_2. Butylated hydroxyanisole was added to all TLC solvents to minimize oxidation.

Gas liquid chromatographic (GLC) analyses of the triglycerides as their fatty acid methyl esters were carried out as previously described (11). Methyl esters of fatty acids from the triglycerides, diglycerides, and monoglycerides were prepared by transesterification using the method of Shehata et al. (15). Methyl esters were prepared from the phosphatidyl phenols by transesterification with sodium methoxide using a method adapted from that of Christopherson and Glass (16). A 1-4 mg sample of lipid dissolved in 60 μl light petroleum (bp 50-60 C) was placed in a Kontes reaction vial and 4 μl 2M-sodium methoxide added. The vial was capped securely and gently rotated for 5 min to facilitate mixing. After standing for a further 5 min, 1-8 μl of the light petroleum layer was injected into the GLC.

RESULTS AND DISCUSSION

Application of Stereospecific Analysis to Bovine Milk Fat

Pancreatic lipase hydrolyses triglycerides containing 4:0 in preference to those containing longer chain fatty acids (17) and consequently acceptable data for the positional distribution of fatty acids in bovine milk fat are more difficult to obtain (18). The standard pro-

cedures for stereospecific analysis could, however, be applied to the high molecular weight fraction obtained by silicic acid column chromatography as this fraction contained no detectable butyric acid (4:0). Furthermore, partial deacylation with a Grignard reagent, used in standard procedures, is not suitable to provide diglycerides from low and medium molecular weight triglycerides containing 4:0 in the 3-position, due to the difficulty of separating the 1,2(2,3)-diglyceride and the 1,3-diglyceride products (8). However, with the narrower range of triglyceride species present in the low and medium molecular weight triglyceride fractions, pancreatic lipase gives relatively representative 1,2(2,3)-diglycerides over short reaction times (8). A check on the reliability of the procedures adopted was obtained by comparing the fatty acid compositions of the experimentally produced diglycerides with those obtained by calculation (19) (Table I). With the exception of 4:0 in the low molecular weight fractions and 6:0 in the medium molecular weight fractions, proportions of major components (> 10% of the total) obtained by analysis and by calculation showed deviations of less than 2%. Values of 4:0 obtained by analysis were 16-17% higher in the fractions of low molecular weight and values for 6:0 were 17-19% higher in the fraction of medium molecular weight than obtained by calculation (Table I). In view of possible errors arising from the above-mentioned shortcomings of the procedures, the fatty acid composition at position 3 has been determined by two alternative methods of calculation (Table II). Notwithstanding the different values obtained, either method gave a clear indication of the specific nature of the placement of the major fatty acids between the three positions.

Gel filtration seems to be a less satisfactory alternative to silicic acid column chromatography for the fractionation of bovine milk triglycerides because the proportion of 4:0 in the fraction of highest molecular weight contained 7.2% 4:0, which was very little lower than the 8.1% 4:0 present in the total triglycerides (20). Moreover, those workers found that the nature of the fractionation did not permit the stereospecific analysis of two further fractions of lower molecular weight.

Effects of Elevated 18:2 on Stereospecific Distribution

A comparison of the stereospecific distribution of the fatty acids in the high molecular weight fractions of the two milk fats showed that increases in the levels of 18:2 in each of the three positions were paralleled by decreases in the levels of 14:0 and 16:0 (Table II). The presence of 18:2 in the triglycerides altered the proportional distribution of 14:0 and 16:0 in favor of position 1 at the expense of position 3 (Table III). It would seem that the preference of 18:2 for position 3 over position 1 has the effect of diverting the available 14:0 and 16:0 into position 1. On the other hand, there were identical proportions of 14:0 and 16:0 in position 2 in the triglycerides of both 18:2-rich and normal milk fat. The amounts of 18:0 and 18:1 were hardly altered in any of the three stereospecific positions (Table II) and, consequently, the distribution of these two fatty acids remained unaltered (Table III). Although the trends of distribution of 18:0 and 18:1 were similar to those found by Breckenridge and Kuksis (6) for a high molecular weight distillate, a higher degree of symmetry was found in the distribution of 18:0 in the high molecular weight fraction isolated by silicic acid column chromatography. This may be attributed to a more distinct fractionation of milk fat on silicic acid judged by the absence of 4:0 and the lower 6:0 level. A fraction of high molecular weight obtained by gel filtration exhibited an approximately random distribution of 18:0 and 18:1 (20), but this fraction contained 7.2% 4:0 and is clearly not comparable to the high molecular weight fraction obtained by silicic acid column chromatography. Moreover, the use of the standard procedure of stereospecific analysis (13) with this fraction would have been subject to the same errors as with total milk triglycerides. However, there was agreement in other characteristic features, e.g., the highest proportion of 18:2 was incorporated at position 3, in the high molecular weight fraction in milk triglycerides from cows fed protected 18:2.

Because of the predominance of short chain fatty acids in position 3 of the triglycerides of the low molecular weight fraction, it follows that the effect of increased 18:2 content was restricted to the fatty acids in positions 1 and 2. Linoleic acid was a major component of the fatty acids at both positions 1 and 2 (Table II) in this fraction of the 18:2-rich milk fat, with 18:2 showing a slight preference for position 2 (Table III). Oleic acid was preferentially esterified at position 1 of both milk fats, but the 18:2-rich milk fat tended to have a higher proportion of the 18:1 in position 3 and a lower proportion in position 1 than the control milk fat. The elevated 18:2-content led to large decreases in the amounts of 14:0 and 16:0 in position 2 and of 16:0 in position 1 (Table II). However, as observed in rabbit adipose tissue (21), changes in the proportional distribution

TABLE II

Positional Distribution of Fatty Acids in the Milk Triglycerides and Milk Triglyceride Fractions from Monozygous Twin Cows Fed a Control Diet or a Similar Diet Supplemented with Protected 18:2

Milk fat	Mol wt fraction	Position[a]	Fatty acid composition (mole %)															
			4:0	6:0	8:0	10:0	10:1	12:0	14:0	14:1	15:0	16:0	16:1	17:0	18:0	18:1	18:2	18:3	20:1
		Total	---	0.4	1.1	3.8	---	4.0	13.3	0.7	1.3	27.3	1.9	0.9	16.4	26.5	1.7	0.7	---
		1	---	---	1.7	3.6	---	4.0	8.2	0.4	1.9	29.1	1.7	0.6	18.9	27.2	1.5	1.1	---
Control	High	2	---	---	0.6	3.0	---	5.9	24.4	1.0	1.9	36.9	2.3	0.6	7.2	14.4	1.2	0.6	---
		3[b]	---	2.2	2.6	7.6	---	4.7	6.2	3.0	1.7	15.5	0.9	0.6	18.6	34.2	0.8	1.4	---
		3[c]	---	1.2	1.0	4.8	---	2.1	7.3	0.7	0.1	15.9	1.7	1.5	23.1	37.9	2.4	0.4	---
		Total	---	1.1	1.5	3.5	---	3.2	8.8	0.6	0.7	16.2	1.4	0.7	18.6	26.5	16.1	1.1	---
		1	---	—	0.3	1.3	0.1	2.5	8.0	0.4	1.3	21.8	2.4	1.5	22.0	25.7	11.7	1.0	---
18:2-rich	High	2	---	---	1.6	4.7	---	6.2	16.4	0.2	0.6	23.3	1.7	0.3	9.8	18.4	16.0	0.8	---
		3[b]	---	2.8	1.6	1.7	0.2	1.6	2.8	0.4	2.0	7.1	1.5	1.3	21.0	33.6	18.6	3.8	---
		3[c]	---	3.3	2.6	4.5	0.1	0.9	2.0	1.2	0.2	3.5	0.1	0.3	24.0	35.4	20.6	1.4	---
		Total	5.3	10.4	4.3	5.9	0.5	4.5	11.8	1.4	1.0	23.6	2.1	0.8	11.1	15.3	5.0	0.8	0.2
		1	---	---	0.7	0.9	---	2.9	9.8	---	0.4	32.3	1.5	0.3	19.8	25.7	2.1	1.9	1.6
Control	Medium	2	---	1.8	3.0	6.1	0.3	7.3	22.6	1.2	1.6	32.1	1.6	0.5	7.2	12.7	1.3	0.7	---
		3[b]	19.0	30.2	7.0	5.3	0.3	2.3	1.8	0.8	0.4	10.1	2.4	0.5	9.6	8.3	1.1	0.7	0.0
		3[c]	15.9	29.4	9.2	10.7	0.2	3.3	3.0	3.0	1.0	6.4	3.2	1.6	6.3	7.5	0.4	0.2	1.0
		Total	8.2	12.3	3.8	4.5	0.3	2.6	7.3	0.4	0.6	14.0	0.9	0.6	15.3	15.9	11.6	0.7	1.0
		1	---	---	0.4	1.9	---	2.8	7.6	---	1.0	19.5	1.6	0.9	28.5	21.4	13.4	0.8	0.2
18:2-rich	Medium	2	---	1.4	4.8	6.7	0.3	5.3	14.8	0.7	0.9	20.4	1.3	0.5	11.6	17.5	13.1	0.9	0.3
		3[b]	24.2	36.0	3.6	2.9	0.1	1.5	1.5	0.1	0.5	8.5	0.5	0.3	8.2	8.1	3.9	0.1	0.1
		3[c]	24.6	35.5	6.2	4.9	0.6	0.3	0.0	0.5	0.1	2.1	0.2	0.4	5.8	8.8	8.3	0.4	2.5
		Total	25.0	6.2	2.3	3.6	0.5	3.8	11.5	0.9	0.9	20.2	1.4	0.4	7.6	12.6	1.2	1.2	0.8
		1	--	---	0.4	1.2	0.1	2.9	10.3	0.2	1.7	34.4	1.6	0.7	19.8	23.5	2.1	0.8	0.4
Control	Low	2	0.1	3.2	5.6	8.9	0.8	9.0	22.3	1.4	2.2	25.5	1.6	0.3	4.8	11.8	1.1	0.9	0.5
		3[b]	73.7	15.0	0.8	0.7	0.0	0.8	2.7	0.0	0.4	6.5	1.4	0.1	2.0	1.0	0.7	0.3	1.5
		3[c]	75.0	15.4	0.9	0.7	0.6	0.5	1.9	1.1	1.2	0.7	1.0	0.2	1.8	2.5	0.4	1.5	1.5
		Total	24.3	5.9	2.3	3.7	0.3	3.4	9.1	0.6	0.6	13.1	1.0	0.2	8.0	13.2	13.5	0.8	---
		1	---	---	1.5	2.6	0.1	3.4	8.7	0.3	2.1	23.3	2.1	0.6	18.9	19.6	15.5	1.3	---
18:2-rich	Low	2	---	5.6	6.3	9.2	0.6	7.5	14.5	0.8	1.0	13.7	1.5	0.3	5.8	13.7	18.5	1.0	---
		3[b]	71.0	9.0	2.1	1.6	0.2	0.9	3.3	0.0	0.6	7.5	1.3	0.5	3.6	3.9	2.5	0.2	---
		3[c]	72.9	12.1	0.9	0.7	0.1	0.7	4.1	0.7	1.3	2.3	0.6	0.3	0.7	6.3	6.5	0.1	---
		Total	11.9	5.3	2.3	4.1	0.3	3.5	11.9	1.0	1.0	22.1	2.0	0.9	11.9	18.6	1.8	0.8	0.5
		1	---	---	0.9	2.0	---	3.3	9.4	0.2	1.5	32.1	1.6	0.6	19.5	25.3	1.9	1.1	0.5
Control	Milk fat	2	---	1.8	3.3	6.2	0.4	7.5	23.1	1.2	2.0	30.9	1.9	0.4	6.1	12.9	1.2	0.9	0.2
		3	36.3	13.2	2.2	3.8	0.3	1.8	3.9	1.3	0.1	8.9	1.6	0.6	9.2	14.9	0.9	0.8	0.1
		Total	10.4	5.4	2.1	3.2	0.2	2.6	7.7	0.5	0.7	14.1	1.1	0.5	14.1	20.6	15.5	0.7	0.7
		1	---	---	0.8	1.9	0.1	2.9	8.2	0.3	1.5	21.9	2.1	1.0	22.1	22.6	13.5	1.1	---
18:2-rich	Milk fat	2	---	2.4	4.0	6.8	0.3	6.5	15.3	0.5	0.8	19.1	1.5	0.3	8.7	16.5	16.4	0.9	0.1
		3	31.8	12.2	1.3	1.7	0.1	0.7	2.6	0.5	0.4	5.2	0.5	0.4	11.6	18.4	11.3	1.2	0.2

[a]Relative to *sn*-glycerol-3-phosphate.
[b]2 x (2,3-PL)-(2-MG).
[c]3 x (TG)-(1-PL)-(2-MG).

TABLE III

Proportional Distribution of Major Fatty Acids in the Milk Triglycerides and Milk Triglyceride Fractions of High, Medium, and Low Molecular Weight of Monozygous Twin Cows Fed a Control Diet or a Similar Diet Supplemented with Protected 18:2

Milk fat	Mol. wt fraction	Position[a]	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	18:1	18:2
		1	---	---	41	28	30	21	36	40	35	35
Control	High	2	---	---	15	23	44	62	45	15	19	28
		3[b]	---	100	44	49	26	17	19	45	46	37
		1	---	---	8	14	26	30	43	41	33	25
18:2-rich	High	2	---	---	40	52	62	61	46	18	23	34
		3[b]	---	100	52	34	13	9	11	41	44	41
		1	---	---	6	6	22	28	44	57	56	57
Control	Medium	2	---	6	25	41	56	65	44	21	27	35
		3[b]	100	94	69	53	22	7	11	23	17	8
		1	---	---	4	15	32	34	43	60	45	41
18:2-rich	Medium	2	---	4	48	54	61	63	45	25	37	40
		3[b]	100	96	48	31	7	4	12	15	18	19
		1	---	---	7	12	24	29	54	80	65	55
Control	Low	2	---	17	93	88	75	64	40	20	33	29
		3[b]	100	83	---	---	1	7	6	---	2	16
		1	---	---	19	22	31	32	56	73	51	40
18:2-rich	Low	2	---	35	81	78	68	54	33	22	36	48
		3[b]	100	65	---	---	1	14	11	5	13	12
		1	---	---	14	17	26	26	45	56	48	48
Control	Milk fat	2	---	12	52	51	60	63	43	18	24	30
		3[b]	100	88	34	32	14	11	12	26	28	22
		1	---	---	13	18	29	31	48	52	39	33
18:2-rich	Milk fat	2	---	16	66	66	64	59	41	21	29	40
		3[b]	100	84	21	16	7	10	11	27	32	27

[a]Relative to *sn*-glycerol-3-phosphate.

[b]Calculated using the average of the two calculations for position 3.

of the major fatty acids arising from the availability of high levels of 18:2 were minor, and the fatty acids remained distributed within the triglycerides in a characteristic manner.

Smith et al. (9) used the pancreatic lipase technique to show an additional introduction of oleic acid and linoleic acid at position 2 in 18:2-rich milk. Stereospecific analysis shows that this arises from a substantial reduction in the proportions of these two fatty acids in position 1 and also shows that the proportions increase in position 3 as well as in position 2. Moreover, it should be emphasized that the order of preference $2 > 1 > 3$ for the positional introduction of linoleic in total milk fat is an average order. The order of preference for 18:2 in the constituent triglycerides of high molecular weight is $3 > 2 > 1$ and that in the constituent triglycerides of medium and low molecular weight is $2 \geqslant 1 > 3$.

Relationship of Bovine Milk Fat to Other Animal Fats

Assessing the extent to which the triglycerides of bovine milk fat follow the trends in the stereospecific placement of fatty acids in animals (1) is complicated by the placement of short chain fatty acids in position 3 in almost 50% of the triglycerides. Consequently, a clear representation of the positional distribution of fatty acids in the remaining constituent triglycerides is obtained only by a separate analysis of the high and low molecular weight triglycerides as conducted in the present study. For example, palmitic acid was preferentially located in position 2 in the high molecular weight triglycerides, with the least present in position 3 (Table III), as has been found in human milk fat (22). However, 16:0 was preferentially located in position 1 in the low molecular weight triglycerides and this resulted in an almost even distribution between positions 1 and 2 in the total milk fat (Tables II and III). Oleic acid was preferentially located in positions 1 and 3 in the high molecular weight triglycerides with only about 20% in position 2 which is very similar to the distribution of 18:1 in human milk fat (22). However, 18:1 was virtually absent in position 3 of the low molecular weight triglycerides and, consequently, a higher proportion was located in positions 1 and 2. Nevertheless, the ratio in these two positions was about the same in the low molecular weight triglycerides as in the high molecular weight triglycerides. Therefore, although the overall distribution of oleic acid in milk fat favored position 1 with the proportion in position 3 exceeding only slightly the proportion in position 2 (Table III), this distribution did not reflect the distribution patterns in the constituent triglycerides. Stearic acid (18:0) had a similar pattern of distribution within the high and low molecular weight triglycerides to that of 18:1.

The distribution of 18:2 in high molecular

weight triglycerides of bovine milk and in human milk triglycerides, containing 16 and 11% 18:2, respectively (22), is reasonably similar in that slightly more than 40% is located in position 3 of the triglycerides. In contrast, 18:2 showed a slight preference for position 2 over position 1 in the low molecular weight triglycerides which leads to the similarities in the distribution of fatty acids of high molecular weight triglycerides of bovine milk and the triglycerides of human milk being overlooked when the distribution of fatty acids in the total triglycerides are compared (22). The triglycerides in the fraction of medium molecular weight which was 20% of the total milk triglycerides, like the milk fat itself, tended to have distributions of the major fatty acids which were averages of the high and low molecular weight fractions.

Changes in Individual Species of Triglycerides

The individual molecular species of triglycerides which undergo the greatest quantitative changes with the presence of elevated levels of 18:2 for esterification may be assessed from the triglyceride composition (11) and the positional distribution of the fatty acids reported here. Decreases in the level of saturated triglycerides such as *sn*-glycerol-1-palmitate-2-myristate-3-stearate and *sn*-glycerol-1,2-dipalmitate-3-stearate or 3-butyrate, monoene triglycerides such as *sn*-glycerol-1-palmitate-2-myristate-3-oleate and *sn*-glycerol-1-oleate-2-palmitate-3-stearate or 3-butyrate, and diene triglycerides such as *sn*-glycerol-1-oleate-2-palmitate-3-oleate appear likely. On the other hand, it may be deduced that there was an increase in the level of diene triglycerides such as *sn*-glycerol-1-stearate-2-palmitate-3-linoleate and *sn*-glycerol-1-stearate-2-linoleate-3-stearate or 3-butyrate, triene triglycerides such as *sn*-glycerol-1-stearate-2-linoleate-3-oleate and *sn*-glycerol-1-oleate-2-linoleate-3-butyrate, and tetraene triglycerides such as *sn*-glycerol-1-stearate-2,3-dilinoleate and *sn*-glycerol-1,2-dilinoleate-3-butyrate.

Biosynthesis of Milk Fat Triglycerides

Similarities in the fatty acid composition in positions 1 and 2 of bovine milk triglycerides of differing fatty acid composition in the position 3 (6) have been cited as evidence for a common pool of 1,2-diglycerides being acceptors for acyl groups in the final step of triglyceride biosynthesis via the glycerol-3-phosphate pathway (6,23). However, an interesting pattern of distribution with respect to 16:0 in positions 1 and 2 of triglycerides has emerged from the fractionations carried out in the present study and in the earlier work of Taylor and Hawke (8). Palmitic acid shows a preference for the 2-position relative to the 1-position in the high molecular weight triglycerides, is evenly distributed in the medium molecular weight triglycerides, and favors the 1-position relative to the 2-position in the low molecular weight triglycerides. Dimick et al. (24) and Barbano and Sherbon (25) also observed a greater relative abundance of 16:0 in position 2 of bovine milk triglycerides of higher molecular weight and higher melting point, respectively. The high proportions of 16:0 in position 2 of the low density lipoproteins of ruminant blood serum (26) and the above data are not inconsistent with the suggestion that a 2-monoglyceride pathway has some role in the biosynthesis of the high molecular weight triglycerides of ruminant milk (24), although metabolic and compositional studies suggest that the glycerol-3-phosphate pathway involving 1,2-diglycerides as intermediates is likely to be the dominant route in the synthesis of bovine milk triglycerides (27).

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Leucine and Isoleucine as in vitro Precursors for Lipid Synthesis by Rat Aorta[1]

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ABSTRACT

The in vitro conversion of ^{14}C-labeled leucine, isoleucine, and pyruvate to specific lipids was compared in rat aorta, diaphragm, and fat pad. Total lipid specific radioactivity from all precursors was greatest in aorta. The ratio of label incorporated into polar lipids vs. neutral lipids by aorta was generally several-fold that incorporated by muscle and fat pad. The labeling of sterols in the aorta from ^{14}C-leucine and pyruvate was equivalent. It is concluded that leucine may be a substantial precursor to polar lipids and to sterols in rat aorta.

INTRODUCTION

Although the liver is generally accepted as the primary locus of amino acid degradation (1), the branched chain amino acids are more readily oxidized by extrahepatic tissues (2-8). This is largely due to the lack of branched chain amino acid transaminase activity in the liver (4). The first three steps in the degradation of branched chain amino acids occur over a common pathway: (a) transamination to the corresponding α-keto derivative, (b) oxidative decarboxylation to the acyl CoA thioester, and (c) dehydrogenation to the α,β-unsaturated acyl CoA thioester (9). Based on whole body contribution, muscle and fat pad have been estimated to be the primary sites of the oxidation of these essential amino acids (7). Kidney, brain (10), and aorta (11) have very high capacities to oxidize leucine.

Leucine is a precursor to β-hydroxy-β-methylglutaryl CoA, an intramitochondrial intermediate which is a precursor to sterols by the generation of acetoacetate and acetyl CoA. Leucine has been proposed as a major source of serum cholesterol (12), and muscle (7,12) and fat pads (7) have been suggested as major sites for cholesterol biosynthesis.

The concentration of the branched chain amino acids in plasma is increased during fasting (8,13) and in uncompensated diabetes (14,15) in humans and rats. Circulating branched chain amino acids have been reported to be elevated in obese man (16). The oxidation of branched chain amino acids is regulated by the nutritional state (8,10,17) and by hormones (17). Diaphragms (skeletal muscle) (18) and sciatic nerves (19) from diabetic rats produce more $^{14}CO_2$ from [^{14}C-1] leucine in vitro than control tissue. It is possible that in altered metabolic states, leucine and to a lesser degree, isoleucine may play increased roles as precursors of certain lipids. We undertook this study to ascertain the comparative degree to which ^{14}C-labeled leucine, isoleucine, valine, and pyruvate serve in vitro as precursors to lipids in rat aortas, diaphragm, and fat pad. Pyruvate was chosen as a non-amino acid precursor because initially it is catabolized by virtually the same mechanism as the branched chain amino acids, oxidative decarboxylation and conversion to a CoA thioester in mitochondria.

METHODS

Male Wistar rats weighing 200-250 g were fed ad lib. Wayne Lab-Blocks (Allied Mills Inc., Chicago, IL) and water. Rats were decapitated with a guillotine (Harvard Apparatus Co., Dover, MA), and tissues were immediately excised. The two hemidiaphragms of each rat were dissected separately (6). The epididymal fat pads were excised and divided into six to eight fragments. The descending aorta was opened via a longitudinal incision; the adventitia and perioaortic tissues were stripped off manually, and the aortic strips were divided into halves.

The excised tissues were placed in Gey and Gey's balanced salt solution (20) at room temperature until sufficient tissue was collected for incubation of pooled samples. Before incubation, the tissues from each rat were equally distributed into two incubation media containing different ^{14}C-precursors. Three ^{14}C-precursors were tested in most experiments. The distribution of tissues was randomized. Each incubation vessel contained pooled aortic tissue from three to four rats or pooled muscle or fat pad from two rats. The range in weights of pooled tissues and volumes of incubation medium were: diaphragm 0.6-0.9

[1]This paper was presented in part at the 1974 Biochemistry and Biophysics Meeting of the Fed. Am. Soc. Exp. Biol.

TABLE I

In vitro Incorporation of 14C Labeled Leucine, Isoleucine, and Pyruvate into Total Lipids of Rat Aorta, Diaphragm, and Fat Pad[a]

14C-precursor	Tissue		
	Aorta	Diaphragm	Fat pad
	(dpm/mg tissue/3 hr)		
Leucine	79.9 ± 16.8[b]	17.3 ± 3.2	88.3 ± 16.4
Isoleucine	39.5 ± 6.2	12.0 ± 2.0	93.7 ± 22.4
Pyruvate	273 ± 48.0	76.4 ± 14.6	478 ± 86.5

[a]Rat tissues were incubated in Gey and Gey's balanced salt solution containing 5.5 mM glucose and 0.1 mM of the ^{14}C precursor in an atmosphere of 95% O_2 + 5% CO_2, at 37 C, and pH 7.4.

[b]Standard error. Each mean represents 5-7 observations.

g in 6 ml, fat pad 1.0-2.5 g in 6 ml, and aorta 50-70 mg in 3 ml. Approximately 30 min elapsed between sacrifice and the start of incubation. Tissues were incubated in sealed Erlenmeyer flasks in a Dubnoff metabolic shaker (42 cycles/min). The incubation medium consisted of 5.5 mM glucose and 0.1 mM ^{14}C-labeled precursor in Gey and Gey's balanced salt solution (20), equilibrated with O_2 + CO_2 (95:5), pH 7.4.

Following 3 hr incubation at 37 C, the tissues were blotted dry on filter paper, placed in glass-distilled chloroform-methanol (2:1), and extracted by the method of Folch et al. (21). Water was added, the phases were separated, and extracts were washed three times with upper phase (21). The lower phase was evaporated to dryness with a stream of nitrogen in a water bath at 50 C, and the lipid residues were weighed. Tissue dry weights were determined gravimetrically after drying the lipid-extracted tissue segment in an oven at 100 C for 24 hr. Nonlipids were counted in a liquid scintillation spectrometer (Packard Tricarb, Model 574) after solubilization in 0.5 ml of 2N NaOH. The scintillation fluid consisted of 1.5 ml of Biosolve (Beckman Instruments, Inc., Palo Alto, CA) in 10 ml of toluene containing 5 g/l PPO (2,5-diphenyloxazole) and 0.3 g/l dimethyl-POPOP (1,4-bis-2-(4-methyl-5-phenyloxazolyl-benzene).

Neutral lipids consisting of cholesteryl esters, triglycerides, free fatty acids, diglycerides, and free sterols were separated by thin layer chromatography (TLC) of total lipids on silica gel (Supelcosil 12A, Supelco, Inc. Bellefonte, PA). Polar lipids remained at the origin. Standard lipid mixtures containing hydrocarbons, sterol esters, methyl esters, free fatty acids, free cholesterol, fatty alcohols, and diglycerides were spotted on both edges of plates. Development was performed in petroleum ether-ethyl ether-acetic acid (70:30:0.5), and bands were visualized by spraying lightly with 2′,7′-dichlorofluorescein and viewing under ultraviolet light. Bands were quantitatively transferred to counting vials and counted in a liquid scintillation spectrometer. The scintillation fluid was the same as that described above. Sample quenching was monitored with internal and external standards and was not variable.

In order to insure that ^{14}C counts recovered in the free sterols were not vitiated by ^{14}C free fatty acids, [^{14}C] palmitic acid (10 μCi) and [^{3}H] cholesterol (25 μCi) were added to a chloroform-methanol extract and carried through the entire procedure. Less than 0.05% of [^{14}C] palmitate was recovered in the free sterol band. As expected, we found that the sterol fraction resolved by this TLC method was contaminated by quantitatively small amounts of diglycerides of very high specific radioactivities. Only the diaphragm diglycerides were visible as a poorly resolved band above cholesterol after spraying. This band co-chromatographed with 1,3-dipalmitoyl glycerol and disappeared upon mild alkaline hydrolysis (22).

In order to remove radioactivity due to the presence of diglycerides, aliquots of lipid extracts containing 2 to 8 mg of ^{14}C-labeled total lipids and an added internal standard of [^{3}H] cholesterol of known radioactivity were deacylated in 2 ml of 0.2 N methanolic NaOH overnight at room temperature in Teflon-lined screw-cap vials. The mixture was extracted three times with 2 ml of petroleum ether. The combined extracts were evaporated with a stream of nitrogen to a convenient volume and were separated by TLC as described above for neutral lipids. All tabulated values for ^{14}C incorporation into sterols have been corrected for recovery according to the amount of [^{3}H] cholesterol recovered. The overall recovery of sterols was 80 ± 1.0% (SEM, 99 observations). Because values for incorporation of radioac-

TABLE II

In vitro Incorporation of ^{14}C from Labeled Leucine, Isoleucine, and Pyruvate into Lipid Classes of Rat Aorta[a]

Lipid class[b]	^{14}C-precursor Leucine	Isoleucine	Pyruvate
		(dpm/mg)	
Sterols	7.4 ± 0.8[c]	1.8 ± 0.4	6.6 ± 1.4
Triglycerides	41.0 ± 4.3	20.0 ± 3.4	205.3 ± 4.4
Phosphatidyl choline	18.1 ± 1.6	11.5 ± 2.4	30.0 ± 4.6
Phosphatidyl ethanolamine	2.7 ± 0.3	1.8 ± 0.4	5.5 ± 0.8
Total neutral lipids	56.3 ± 2.2	25.3 ± 2.8	234.8 ± 5.5
Total polar lipids	23.5 ± 2.2	14.2 ± 1.6	38.2 ± 2.1

[a]Tissue segments were incubated as described in Table I.

[b]Other lipid classes were analyzed but each contained less than 2% of the radioactivity. They were: free fatty acids, cholesteryl esters, phosphatidyl serine, sphingomyelin, phosphatidic acid, lysophosphatidyl choline, lysophosphatidyl ethanolamine, and several unidentified lipids. The diglycerides accounted for 6-8% of the radioactivity.

[c]Standard error. Each mean represents 5-7 observations.

tivity into diglycerides were not obtained by a direct method they were not tabulated, but estimates are included in the footnotes of each table. The contribution of ^{14}C-label to the individual sterols of the sterol pool was not determined.

Polar lipids were separated by the method of Rouser et al. (23) on silica gel containing 10% magnesium silicate (Supelcosil 42A). Radioactivity in individual spots was determined as described above for neutral lipids.

Uniformly labeled L-leucine, L-isoleucine, L-valine, and sodium [^{14}C-2] pyruvate were purchased from New England Corp., Boston, MA. The concentration of radioactivity used in the incubation medium was 0.2 μCi/ml.

Means ± the standard errors of the means are tabulated. The significance of the difference between means was tested by Student's t-test.

RESULTS AND DISCUSSION

In initial experiments, the incorporation of ^{14}C into total lipids from leucine, isoleucine, and valine was compared. Minimal amounts of label from valine were incorporated into aortic lipids, and the same behavior was observed in diaphragm and fat pad, confirming the finding of Rosenthal et al. (7). No further experiments with labeled valine were therefore performed. The incorporation of label into whole tissues and into total lipids from leucine and pyruvate was measured in all tissues after 1, 2, and 3 hr of incubation, and the incorporation of label was linear.

In Table I, it is shown that the incorporation of label from [^{14}C] leucine into lipids per unit weight of aorta was comparable to that observed in fat pad and about fourfold that in diaphragm. Additionally, the leucine/isoleucine incorporation ratio in aorta was 2:1 ($p<.05$), and the leucine/pyruvate ratio was 1:3 ($p<.01$). In contrast, the leucine/isoleucine ratios observed in the diaphragm and fat pad were close to unity, and the leucine/pyruvate ratios in these tissues were lower than those found in the aorta. Counts incorporated into total lipids from leucine were not only roughly one-third those incorporated from pyruvate, but also twice those incorporated from isoleucine, phenomena not observed in either diaphragm or fat pad. These data infer that leucine is a substantial in vitro lipid precursor in the aorta.

The incorporation of label into lipids on a tissue weight basis (dpm/mg tissue) is dependent on the lipid content, and largely because of this the incorporation of label into aortic lipids appeared relatively low in Table I. In contrast, the specific radioactivity of aortic lipids (dpm/mg lipid) was much higher than that observed in diaphragm and fat pad, irrespective of precursor. The leucine/isoleucine and leucine/pyruvate incorporation ratios within each tissue were comparable to those shown in Table I, but the incorporation of label from [^{14}C] leucine into aortic lipids was 10-fold that seen in diaphragm and nearly 20-fold that in fat pad. Because the lipid contents of aorta and diaphragm were almost identical, the higher specific radioactivities observed in aortic lipids cannot be explained in terms of lipid content. The nonlipids of aorta were also labeled to a greater relative degree than those of diaphragm or fat pad. It was calculated that in the 3 hr incubation, the aorta incorporated ca. 4-10 times more ^{14}C from leucine into nonlipids than did the other tissues. This indicates that, compared to the other tissues tested, the intima and/or media of

TABLE III

In vitro Incorporation of ^{14}C from Labeled Leucine, Isoleucine, and Pyruvate into Lipid Classes of Rat Diaphragm[a]

Lipid class[b]	^{14}C-precursor Leucine	Isoleucine	Pyruvate
		(dpm/mg)	
Sterols	0.3 ± 0.01[c]	0.1 ± 0.01	0.5 ± 0.1
Triglycerides	10.5 ± 0.4	7.8 ± 0.1	55.1 ± 1.1
Phosphatidyl choline	1.4 ± 0.3	0.7 ± 0.1	5.1 ± 5.1
Phosphatidyl ethanolamine	0.3 ± 0.1	0.1 ± 0.01	1.3 ± 0.7
Total neutral lipids	14.8 ± 2.3	10.9 ± 2.1	69.7 ± 6.7
Total polar lipids	2.5 ± 0.3	1.1 ± 0.1	6.5 ± 0.7

[a]Tissue segments were incubated as described in Table I.
[b]Other lipid classes were analyzed but were found to contain less than 1% of the radioactivity. They were: free fatty acids, cholesteryl esters, phosphatidyl serine, sphingomyelin, phosphatidic acid, lysophosphatidyl choline, lysophosphatidyl ethanolamine, diphosphatidyl glycerol, and several unidentified lipids. Radioactive diglycerides accounted for 14-20% of the radioactivity.
[c]Standard error. Each mean represents 5-7 observations.

TABLE IV

In vitro Incorporation of ^{14}C from Labeled Leucine, Isoleucine, and Pyruvate into Lipid Classes of Rat Fat Pad[a]

Lipid class[b]	^{14}C-precursor Leucine	Isoleucine	Pyruvate
		(dpm/mg)	
Sterols	0.39 ± 0.03[c]	0.26 ± 0.01	1.1 ± 0.1
Triglycerides	69.1 ± 2.0	70.5 ± 2.4	416 ± 26.7
Total neutral lipids	79.8 ± 7.1	87.0 ± 9.0	453 ± 32.1
Total polar lipids	8.4 ± 1.1	6.7 ± 0.9	24.4 ± 3.9

[a]Tissue segments were incubated as described in Table I.
[b]Radioactivity in free fatty acids and cholesteryl esters was less than 1% of the total. Polar lipid subclasses were not analyzed because of the paucity of polar lipids in this tissue. Diglycerides accounted for 7-16% of the radioactivity.
[c]Standard error. Each mean represents 4-6 observations.

aorta are very active metabolically.

In order to account for the labeling patterns observed in total lipids, the incorporation of label into specific lipid classes was studied and is shown in Tables II, III, and IV. It is clear that in all cases, the largest fraction of label appeared in the neutral lipids (cholesteryl esters, triglycerides, free fatty acids, diglycerides, and free sterols), and most of the label was in the triglyceride fraction. Label from pyruvate accounted for the greatest fraction of label in the neutral lipids in each tissue. In aorta (Table II), the ratios of incorporation into neutral vs. polar lipids were much lower than in diaphragm (Table III) or fat pad (Table IV), especially when leucine and isoleucine were the labeled precursors. These observations suggest that relative to other tissues, polar lipid turnover or synthesis in the aorta was more rapid relative to the neutral lipids, and that the precursor pools may not have been homogeneous. Of the polar lipids, phosphatidyl choline contained the largest fraction of label. Seth and Newman (24) demonstrated in rabbits that of the aortic phospholipids, phosphatidyl choline was the most rapidly synthesized component when [^{14}C] acetate was used as a precursor. Our data show that leucine and isoleucine may also be substantial precursors to aortic polar membrane lipids.

The comparative incorporation of ^{14}C into aortic sterols from the three precursors was studied. In Table II, it is clear that the labeling of sterols resulting from leucine and pyruvate was nearly equal, but the incorporation from isoleucine was less. This pattern of labeling was not observed in diaphragm or fat pad where much less label from leucine was contributed to sterols. Our data support the findings of Rosenthal et al. (7) and Seth and Newman (24) who reported cholesterol synthesis from [^{14}C] leucine by fat pad and muscle. Further, our data suggest that leucine may be a substantial in situ

precursor of cholesterol in the rat aorta. To our knowledge, this is the first such observation in aorta. Miettinen and Penttila (12) found leucine to be a better cholesterol precursor than acetate in homogenates of rat quadriceps femoris muscle. In vitro the rat aorta oxidizes leucine more rapidly than rat diaphragm (11), inferring that leucine could be a substantial precursor for metabolic intermediates required in cholesterol biosynthesis.

Since the relative rates of synthesis and degradation of specific lipids and the sizes of precursor pools were not determined in this study, the interpretations of the relative labeling patterns observed is somewhat speculative. However, all tissues from individual animals were evenly distributed between incubation media, the concentration of precursors in media was equimolar, the specific radioactivities of precursors were the same, and incorporation of ^{14}C was linear. It is attractive, therefore, to attribute the relatively high specific radioactivities of lipids and nonlipids in the aorta to the rapid turnover or synthetic rates of these components.

This study suggests that leucine may be a significant precursor to cholesterol in the aorta. This finding may be especially relevant in conditions such as obesity and diabetes mellitus, which are associated with an increased incidence of atherosclerosis and increased circulatory levels of leucine (14,15,17). Although plasma cholesterol is probably the major source of cholesterol in atheromatous rabbit aorta (25), the possible role of leucine as an in situ source deserves further study. While skeletal muscle of diabetic rats oxidizes leucine faster than that of controls (18), no similar studies have been reported with smooth muscle. Such a mechanism in smooth muscle could increase the aortic supply of catabolic products from leucine, i.e., β-hydroxy-β-methylglutaryl CoA, acetoacetate, and acetyl CoA, to contribute substantially to the in situ synthesis of sterols.

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Effect of Polyunsaturated Fatty Acids of the α-Linolenic Series on the Development of Rat Testicles

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ABSTRACT

The effect of 22:6ω3 acid provided by dietary fish oil on the development of germinal tissue of rat testes, fatty acid composition of lipids, and linoleic or α-linolenic acid Δ6 desaturation capacity was investigated. Results were compared to those obtained in animals fed methyl palmitate and sunflower seed oil (linoleate). At 7 and 9 weeks of age, development of germinal tissue of animals fed fish oil was normal. The fatty acid composition showed a decrease in 22:5ω6 acid content and an increase in 22:6ω3 acid in triacylglycerol, phosphatidylcholine, and phosphatidylethanolamine. The fatty acid Δ6 desaturation capacity of testicules microsomes was increased. It is suggested that 22:6ω3 acid may functionally replace 22:5ω6 acid in germinal tissue.

INTRODUCTION

It is evident that polyunsaturated fatty acids of 22 carbon chain length play an important role in animal testicles since they are generally found in these organs in relatively large amounts (1). However, species differences are easily recognized and whereas in human (1) or bull (2) 22:6ω3 acid (α-linolenic acid family) predominates, in other animals as in the rat, rabbit, or dog (1), the 22:5ω6 acid (linoleic acid family) is found. Histological studies done parallel to fatty acid composition analysis during the development of rat testes demonstrate a close correlation between the increase of 22:5ω6 concentration and the appearance of spermatids and spermatoza.

Surgical cryptorchidism or administration of $CdCl_2$ that destroy cellular elements decrease the concentration of this acid (3). Therefore, it was considered that the 22:5ω6 acid is vitally concerned with the germinal cells of the rat organ.

Since the food of laboratory rats generally contains fatty acids of the linoleic acid family and is devoid or practically devoid of the elements of the α-linolenic acid series, it is quite possible that the specificity of rat testes for 22:5ω6 acid and the absence of 22:6ω3 acid may be circumstantial and only the consequence of food composition. For this reason, the effect of three different diets containing corn oil (linoleic acid), methyl palmitate, and fish oil (20:5ω3 and 22:6ω3) on the fatty acid metabolism and maturation of rat testes was investigated in the present work. Maturation of mammalian testes is dependent on the normal proliferation and differentiation of cells of the germinal epithelium. In the rat at 7 weeks of age, Sertoli cells have matured (4) and spermatoza are found within the tubular lumen (5). These events parallel a marked accumulation of 22:5ω6 in the tissue (6). For this reason, it was important to investigate the effect of the three diets at 7 and 9 weeks of age.

EXPERIMENTAL PROCEDURES

Materials

cis-1-^{14}C linoleic acid (58 mCi/mmol, 99% radiochemically pure) and *cis*-1-^{14}C α-linolenic acid (58 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). Unlabeled acids were provided by the Hormel Institute, Austin, MN.

Animals and Diets

Male weanling rats (21-days-old) of the Wistar strain were started on the experimental diets in groups of five. Three types of diets were used. They were based on a fat-free diet containing 17% defatted casein, 77% sucrose, 4% mineral mixture, and 2% vitamin mixture (7). To one of the diets was added 3% methyl palmitate, to another 3% sunflower seed oil (containing 52% linoleic acid), and to the third one 3% fish oil. The fish oil contained only 2% 18:2 acid but 7.5% 20:5ω3 and 17.0% 22:6ω3 (Table I). Six groups of rats were fed each diet. The rats were decapitated at 7 or 9 weeks of age. The weight of the body and testis, the histology of the testis, the fatty acid composition of testicular lipid fractions, and microsomal fatty acid desaturation activity were determined.

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TABLE I

Fatty Acid Composition of Diets[a]
(% of Total Fatty Acids)

Fatty acids	Diets		
	+ Methyl palmitate	+ Sunflower seed oil	+ Fish oil
14:0	3.4	---	4.1
16:0	72.3	20.5	18.1
16:1	---	1.1	6.3
18:0	7.4	4.3	5.0
18:1	10.8	21.0	20.1
18:2	3.0	52.0	2.0
18:3	---	---	1.1
20:1	---	---	6.9
20:4ω6	---	---	1.1
20:4ω3	---	---	5.9
20:5ω3	---	---	7.5
20:5ω6	---	---	0.4
20:5ω3	---	---	2.7
22:6ω3	---	---	17.0

[a]Totals less than 100% because minor components are omitted.

Separation of Microsomes

After removal of the tunica albuginea, the testes were homogenized and microsomes separated by differential centrifugation at 100,000 x g by the procedure described by Peluffo et al. (8).

Assay for Δ6 Desaturation of Fatty Acids

Microsomal desaturation of 1-^{14}C linoleic acid to γ-linolenic acid and of 1-^{14}C α-linolenic acid to octadeca-6,9,12,15-tetraenoic acid was measured by the procedure described previously (9). Two nmole of each labeled acid were incubated for 20 min at 35 C in a total volume of 0.5 ml with 1 mg microsomal protein. NADH, ATP, CoA, and other cofactors were added in concentrations already described (9). After incubation, the distribution of radioactivity between substrate and product was determined by gas liquid radiochromatography of the fatty acid methyl esters in a Packard apparatus equipped with a proportional counter (9).

Lipid Extraction, Fractionation, and Fatty Acid Analysis

Lipids of total testicles after removal of tunica albuginea were extracted by the procedure of Folch et al. (10). Phosphatidylcholine, phosphatidylethanolamine, and triacylglycerols were separated by thin layer chromatography on Silica-Gel G. Chloroform-methanol-water (65:25:4, v/v) and petroleum ether (60-90 C) ethyl ether acetic acid (90:10:1, v/v) were used to separate polar lipids and triacylglycerol, respectively (11). The fatty acid composition of the individual lipids was determined by gas liquid chromatography (GLC) of the fatty acid methyl esters (12). They were analyzed in a Packard apparatus equipped with a flame detector. The column was packed with 10% polyethylene glycol succinate coated on Chromosorb W (80-100 mesh). Identification of the fatty acids in the chromatogram was done by comparison of their retention times with standards using the graphic procedure of the retention times vs. chain length and Ackman's separation factors (13-15).

Microsomal lipids were extracted by the procedure of Folch et al. (10) with chloroform-methanol (2:1, v/v). The fatty acids were converted to methyl esters and analyzed by GLC.

Histological Examination

In all the groups of rats, a portion of each testis was used for histological study. The material was fixed in Bouin's fluid, dehydrated in ethanol, and embedded in paraffin. The sections were stained with hematoxylin and eosin.

The study of the spermiogenesis was done following Leblond and Clermont's classification (16), calling early spermatids those of the phases 1 and 2 and late spermatids those of the phases 3 and 4.

RESULTS AND DISCUSSION

The effect of the three diets on the body weight of the rats is shown in Figure 1. The gain in body weight for the animals given the fish oil diet was significantly higher than that of the group given palmitate. It was also slightly higher than that of animals given the sunflower seed oil diet. The effect of essential fatty acid (EFA) deprivation was significant at 7 and 9 weeks of age.

The effect of the diets on testicular weights was not significantly different for the three diets during the period tested except for the animals fed palmitate. In testes of these rats, there was a significant decrease at 9 weeks of age (Table II).

These results suggest that the fatty acids of fish oil can replace those of sunflower seed oil during the period tested without significant effect on the weight of the body and of the testicles.

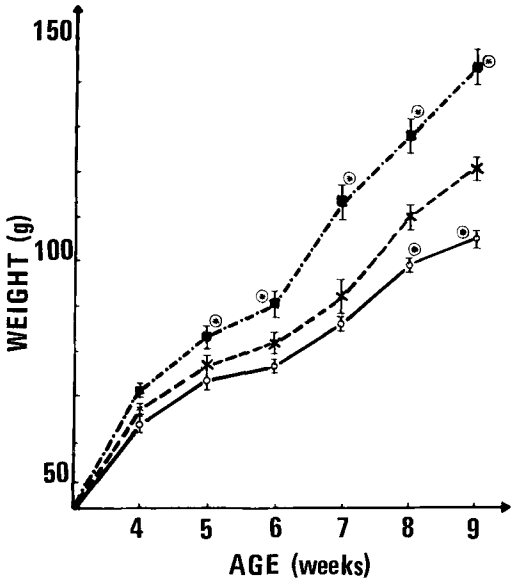

FIG. 1. Growth curve of rats fed on sunflower seed oil (X-------X), methyl palmitate (○———○), and fish oil (■-----■). *Values significantly different of rats fed on sunflower seed oil ($p<0.05$). Bars correspond to standard error of the mean weight of 15 rats.

Fatty Acid Composition of Testes

The fatty acid composition of phosphatidylcholine, phosphatidylethanolamine, and triacylglycerol of testes is given in Tables III-V. It was different for the animals receiving the different diets. The EFA deficient diet containing palmitate evoked a change in the fatty acid composition of the three lipids. In triacylglycerol (Table V), increases in palmitoleate, oleate, and 20:3ω9 acids correlate with decreases in linoleate, arachidonate, and 22:5ω6. However, these changes, typical of EFA deficiency, were not as evident in the phospholipids (Tables III and IV). Whereas linoleate and arachidonate were well depressed and 20:3ω9 was increased, palmitoleate and oleate were not enhanced in the expected way in all the phospholipids analyzed. This result is difficult to explain.

An acid of 20 carbons ($\frac{rt}{rt\ 18:0} = 3.5$) (rt = Retention time) was found in the phospholipids and triacylglycerols of the palmitate-fed animals. The acids, tentatively identified as eicosa-4,7,10,13-tetraenoic acid, belongs to the palmitoleate family and would be synthesized from palmitate by desaturation and elongation reactions. The presence of 20:4ω7 acid originating from palmitoleate had been recognized by Klenk and Olette (17) in phospatides of rats fed a fat-free diet. Sprecher (18) has also shown that 20:4ω7 acid may be synthesized in rat liver from 18:3ω7 and 20:3ω 7 (belonging to the palmitoleate series) when they are administered to EFA deficient rats.

In rats fed sunflower seed oil, the linoleic acid of the diet was converted in the testes to 22:5ω6 by the following sequence of reactions (9,19,20); 18:2 (9,12) $\xrightarrow{\Delta 6\ \text{desaturation}}$ 18:3 (6,9,12) $\xrightarrow{\text{elongation}}$ 20:3 (8,11,14) $\xrightarrow{\Delta 5\ \text{desaturation}}$ 20:4 (5,8,11,14) $\xrightarrow{\text{elongation}}$ 22:4 (7,10,13,16) $\xrightarrow{\Delta 4\ \text{desaturation}}$ 22:5 (4,7,10,13,16).

The 22:5ω6 acid is found both in phospholipids and in triacylglycerol as shown in Tables III-V.

The replacement in the diet of linoleic acid

TABLE II

Weight of Testes in an EFA Deficient Diet

	+ Palmitate		+ Sunflower seed oil		+ Fish oil	
Age	7 Weeks	9 Weeks	7 Weeks	9 Weeks	7 Weeks	9 Weeks
Weight (g)	0.29 ± 0.04[a]	0.35 ± 0.02 $p<0.05$[b]	0.28 ± 0.03	0.47 ± 0.04	0.37 ± 0.03	0.59 ± 0.06

[a]Standard error.

[b]$p<0.05$ referred to sunflower seed oil.

TABLE III

Fatty Acid Composition of Testicular Phosphatidylcholine of Rats Fed Methyl Palmitate, Sunflower Seed Oil, or Fish Oil

Fatty acid[a]	Methyl palmitate		Sunflower seed oil		Fish oil	
	7 Weeks[b]	9 Weeks	7 Weeks	9 Weeks	7 Weeks	9 Weeks
16:0	50.0[c]	53.5	47.1	46.7	47.0	46.8
	(36.0-58.1)	(47.0-59.0)	(42.9-51.4)	(39.1-51.1)	(46.0-47.5)	(44.4-53.1)
16:1	0.9	3.3	1.6	1.8	2.3	2.3
	(0.4-1.3)	(3.0-3.7)	(1.6-1.7)	(1.6-2.5)	(1.4-3.6)	(1.9-3.2)
18:0	9.9	10.2	5.5	6.7	3.8	4.7
	(9.2-10.7)	(8.9-11.6)	(5.3-5.7)	(5.0-10.0)	(3.1-3.9)	(4.1-6.3)
18:1	8.5	11.6	20.9	18.5	28.5	25.6
	(5.9-11.1)	(8.1-15.0)	(18.7-23.1)	(17.1-20.0)	(26.0-30.8)	(24.5-27.3)
18:2	2.8	---	2.8	3.1	0.7	1.2
	(0.6-3.2)		(2.8-2.8)	(2.2-4.1)	(0.6-1.4)	(0.9-1.6)
20:3ω9	1.5	1.1	---	---	0.3	0.7
	(0.5-2.4)	(0.3-2.0)			(0.2-12.0)	(0.4-1.0)
20:4ω6	0.9	1.0	12.4	11.3	9.0	8.0
	(0.4-1.4)	(0.5-2.1)	(11.5-13.4)	(10.3-13.6)	(8.5-10.0)	(5.1-9.9)
20:5ω3	0.1	0.2	0.1	---	1.1	1.0
	(0.1-0.2)	(0.1-0.6)	(0.1-0.1)		(0.7-1.3)	(0.7-1.3)
22:5ω6	5.4	6.0	6.2	7.1	3.3	3.5
	(3.8-6.3)	(4.8-7.0)	(5.7-6.7)	(5.5-8.7)	(2.6-4.5)	(1.8-4.9)
22:6ω3	2.1	2.0	0.4	0.4	2.8	3.3
	(0.3-3.5)	(1.2-2.8)	(0.1-0.7)	(0.2-0.6)	(1.5-4.0)	(2.2-4.1)

[a]Only fatty acids of 16 to 22 carbons were considered. A compound emerging after 20:4ω6 was tentatively identified as 20:4ω7 in an approximate proportion of 10%.

[b]Age of the animal.

[c]Results are the mean of three pools. Range of values in parentheses.

TABLE IV

Fatty Acid Composition of Phosphatidylethanolamine of the Testes of Rats Fed Different Diets

Fatty acid[a]	Methyl palmitate		Sunflower seed oil	Fish oil	
	7 Weeks[b]	9 Weeks	9 Weeks	7 Weeks	9 Weeks
16:0	29.8[c]	38.2	26.6	21.8	22.9
	(29.6-30.0)	(35.2-43.7)	(21.8-29.9)	(17.4-26.1)	(22.2-27.5)
16:1	1.7	1.8	1.5	0.5	1.5
	(1.5-2.0)	(1.4-2.1)	(1.2-1.8)	(0.2-0.6)	(1.4-1.9)
18:0	15.0	12.9	12.5	9.9	9.3
	(13.4-16.6)	(10.9-17.0)	(10.0-15.3)	(9.2-10.5)	(8.0-10.2)
18:1	14.5	15.4	11.9	17.7	17.0
	(10.5-18.4)	(14.3-16.4)	(9.9-13.3)	(13.8-20.9)	(15.4-18.0)
18:2	2.5	1.2	2.5	2.0	2.1
	(2.5-2.5)	(1.1-2.0)	(0.9-3.8)	(1.8-2.4)	(1.8-2.5)
20:3ω9	7.4	5.0	0.9	1.8	1.3
	(4.2-10.6)	(3.8-6.3)	(0.6-1.4)	(0.9-3.5)	(1.3-1.7)
20:4ω6	7.5	7.9	18.7	16.4	16.7
	(4.3-10.7)	(6.7-11.8)	(16.9-20.3)	(16.0-16.9)	(10.5-17.6)
20:5ω3	--	0.6	--	1.2	1.7
		(0.0-1.4)		(1.0-1.2)	(1.5-1.9)
22:5ω6	6.2	8.3	19.9	9.6	8.6
	(3.5-10.0)	(6.3-10.1)	(16.6-24.9)	(8.0-12.2)	(5.3-9.6)
22:6ω3	0.2	0.8	0.6	11.4	10.8
	(0.0-0.4)	(0.4-1.2)	(0.5-0.8)	(7.8-15.3)	(9.5-13.0)

[a]Only fatty acids of 16 to 22 carbons were considered. In animals fed palmitate a compound in approximate concentration of 7.0% was tentatively identified as 20:4ω7.

[b]Age of animals.

[c]Results are the mean of three pools. Range of values in parentheses.

TABLE V

Fatty Acid Composition of Testicular Triacylglycerols of Rats Fed Different Diets

Fatty acid[a]	Methyl palmitate		Sunflower seed oil		Fish oil	
	7 Weeks[b]	9 Weeks	7 Weeks	9 Weeks	7 Weeks	9 Weeks
16:0	35.0[c] (34.0-36.5)	32.9 (25.2-37.8)	33.9 (32.9-34.9)	31.7 (29.9-32.6)	37.8 (36.7-40.6)	39.1 (37.5-40.3)
16:1	11.8 (10.1-14.2)	11.9 (9.4-13.0)	8.9 (7.7-10.2)	8.0 (7.2-9.0)	11.0 (9.0-11.8)	11.8 (10.5-12.6)
18:0	5.4 (4.8-6.1)	5.0 (4.7-5.6)	4.9 (4.8-5.0)	4.7 (4.6-5.0)	4.9 (4.8-5.2)	6.1 (5.7-6.6)
18:1	37.5 (33.8-39.3)	38.0 (30.6-41.5)	30.9 (30.3-31.6)	26.0 (23.9-27.3)	29.8 (26.0-33.3)	30.0 (28.7-31.2)
18:2	1.6 (1.2-2.4)	1.2 (1.0-1.7)	12.0 (10.6-13.5)	12.4 (11.7-13.2)	1.5 (0.4-2.1)	1.9 (1.3-2.4)
20:3ω9	0.3 (0.1-1.0)	0.7 (0.3-1.3)	--	--	--	--
20:4ω6	0.5 (0.3-0.8)	0.6 (0.1-1.3)	1.1 (0.4-1.9)	1.1 (0.6-2.1)	0.5 (0.3-0.7)	0.3 (0.2-0.4)
20:5ω3	0.3 (0.2-0.6)	0.6 (0.5-0.9)	--	--	0.5 (0.2-0.6)	1.4 (1.2-1.8)
22:5ω6	1.7 (0.7-2.5)	3.3 (3.0-3.5)	4.4 (3.9-4.9)	7.0 (5.3-10.3)	3.0 (2.4-3.7)	2.6 (1.6-3.1)
22:6ω3	0.5 (0.4-0.6)	0.6 (0.4-0.9)	0.2 (0.1-0.5)	0.1 (0.1-0.2)	3.9 (3.4-4.5)	2.2 (2.0-2.8)

[a]Only fatty acids of 16 to 22 carbons were calculated. In rats fed palmitate a compound emerging after 20:4ω6 was tentatively identified as 20:4ω7.

[b]Age of animals.

[c]Results are the mean of three pools. Range of values in parentheses.

TABLE VI

Effect of Diet Composition on the Δ6 Desaturation of 1-^{14}C Linoleic and 1-^{14}C α-Linolenic Acid to γ-Linolenic and Octadeca-6,9,12,15-tetraenoic Acid, Respectively, by Microsomes of Rat Testes

Substrates	EFA deficient diet (% Conversion)					
	+ Palmitate		+ Sunflower seed oil		+ Fish oil	
	7 Weeks	9 Weeks	7 Weeks	9 Weeks	7 Weeks	9 Weeks
18:2	3.9 ± 0.6[a] P<0.02	5.3 ± 0.3 P<0.01	1.8 ± 0.1	2.2 ± 0.1	3.8 ± 0.4 P<0.01	3.4 ± 0.1 P<0.01
18:3	8.0 ± 0.1 P<0.01	16.8 ± 0.2 P<0.01	5.0 ± 0.1	11.0 ± 0.2	6.8 ± 1.6	12.4 ± 1.0 P<0.02

[a]Standard error of the mean. P values with reference to sunflower seed oil.

of sunflower seed oil by polyunsaturated acids of the α-linolenic acid family of fish oil evoked typical changes in the fatty acid composition of the testes. They produced, in general, a decrease in linoleic, arachidonic, and 22:5ω6 acids together with an increase in palmitoleate and oleate. However, the accumulation of 22:6ω3 acid provided by the fish oil was the most prominent result. The incorporation was produced in both phospholipids and triacylglycerols but was especially prominent in phosphatidylethanolamine. Of the three lipids studied, phosphatidylethanolamine is the richest in highly polyunsaturated fatty acids, particularly in 22:5ω6 acid. Fish oil also contains 20:5ω3 acid, but this acid is incorporated into the testes only to a limited extent.

Coniglio et al. (21) have shown that testes of rats fed cod liver oil incorporated 20:5ω3 but accumulated 22:6ω3 to a greater degree. In testes of these rats, there was a decrease in 22:5ω6 content.

Carpenter (22) has also found a combined accumulation of 22:5ω6 and 22:6ω3 in the testes of rats fed a mixed diet that contained cod liver oil and other fats. Therefore, both acids, 22:5ω6 and 22:6ω3, were similarly incorporated into the same lipids.

Δ6 Desaturation Capacity of Testes

The effect of three types of diets on the

TABLE VII

Fatty Acid Composition of Microsomes of the Testes of Rats Fed Different Diets

Fatty acid[a]	Methyl palmitate		Sunflower seed oil		Fish oil	
	7 Weeks[b]	9 Weeks	7 Weeks	9 Weeks	7 Weeks	9 Weeks
16:0	36.3[c]	39.2	33.4	40.2	38.2	42.7
	(34.5-37.9)	(30.0-45.4)	(31.2-36.3)	(38.2-42.1)	(35.1-42.4)	(31.3-48.5)
16:1	3.0	4.9	1.9	1.9	2.8	4.4
	(2.8-3.1)	(3.2-7.8)	(1.5-2.2)	(1.3-2.5)	(0.2-3.6)	(3.5-4.9)
18:0	11.5	14.0	11.3	14.8	9.2	13.9
	(9.6-14.0)	(12.5-15.7)	(10.2-13.6)	(13.4-16.1)	(9.0-9.4)	(11.1-17.2)
18:1	20.0	23.5	15.5	16.2	21.5	17.4
	(19.3-20.9)	(21.5-25.8)	(15.1-16.0)	(15.2-17.1)	(20.3-22.7)	(17.1-18.0)
18:2	0.4	1.1	4.4	3.5	2.4	1.6
	(0.4-0.4)	(0.9-1.4)	(3.0-6.9)	(3.2-3.8)	(2.0-3.0)	(1.2-1.9)
20:3ω9	3.5	4.9	--	--	0.5	0.5
	(2.8-4.6)	(2.2-9.0)			(0.4-0.6)	(0.3-0.8)
20:4ω6	7.3	4.0	13.6	10.4	8.8	6.9
	(6.7-8.1)	(2.5-4.9)	(12.1-15.0)	(9.8-11.1)	(8.5-9.0)	(5.3-8.2)
22:5ω6	7.1	1.5	12.9	7.3	4.3	2.6
	(6.7-7.9)	(0.6-3.5)	(10.9-15.5)	(6.9-7.6)	(4.1-4.6)	(1.7-3.6)
22:6ω3	1.9	1.6	0.8	0.3	4.7	4.5
	(1.5-2.4)	(0.2-3.1)	(0.7-0.8)	(0.3-0.3)	(4.4-4.9)	(3.0-8.1)

[a]Only fatty acids of 16 to 22 carbons were considered. A compound, tentatively identified as 20:4ω7, was present in a concentration of 0.7% in the palmitate group.

[b]Age of animal.

[c]Results are mean of three pools. Range of values in parentheses.

fatty acid desaturation capacity of rat testicular microsomes is presented in Table VI. It has been shown by Castuma et al. (23) and Peluffo et al. (24) that in male rats fed an EFA free diet, there is an increase in the Δ6 desaturase activity of liver microsomes. Table VI shows that this also happens in testicles. In animals fed methyl palmitate as the only lipid source, there was a significant increase ($p < 0.01$) in the capacity of testicular microsomes to desaturate at C-6 both 1-^{14}C linoleic and 1-^{14}C α-linolenic when compared to animals fed sunflower seed oil. Testes of animals receiving fish oil also had enhanced capacity to desaturate at Δ-6 both labeled acids when compared to animals fed sunflower seed oil. Moreover, the effect was less pronounced when compared to animals fed an EFA deficient diet. However, we must point out that liver microsomes of rats receiving the fish oil did not react in the same way, and they decreased the Δ6 desaturase activity (25). The different behavior of the two tissues is difficult to explain. It could be related to a different compartmentalization of 22:6ω3 and 22:5ω6 acids in each organ. This difference is in some ways logical, if we consider that 22:5ω6 acid is practically absent in liver, whereas it is a major component of testes.

The fatty acid composition of testicular microsomes is shown in Table VII. It is apparent that in animals fed fish oil there is an accumulation of 22:6ω3 acid, but the proportion of other fatty acids resembles that of the group fed methyl palmitate. They both differ from animals fed sunflower seed oil in the increase in palmitoleic and oleic acids and in the decrease in fatty acids of the linoleate family. These results agree with data of Coniglio et al. (21) published for total fatty acids of animals fed cod liver oil. Therefore, it is logical to think that, in the testes of our experiments, the increase in Δ6 desaturation activity that would also be accompanied by an increase of Δ9 desaturation, is due to a decrease in the supply of fatty acids of the linoleic acid series.

However, the increase in Δ6 desaturation activity of testicles of rats fed fish oil compared to animals fed sunflower seed oil found in our experimental conditions was produced in spite of the incorporation of 22:6ω3 acid, a compound that has been demonstrated to be an inhibitor of Δ6 desaturation in experiments in vitro (26) and in liver in vivo (25). Coniglio et al. (21), after injecting 1-^{14}C linoleic acid to the testes of rats fed cod liver oil, also suggested that ω3 acids inhibit the conversion of linoleate to arachidonate. Therefore, the increase of the Δ6 desaturase found in testes in this experiment is difficult to explain.

Histology of the Testes

The histological examination of rat testes (Fig. 2) demonstrates that animals 7 weeks of age have begun to show the effect of diet composition on the development of the germinal

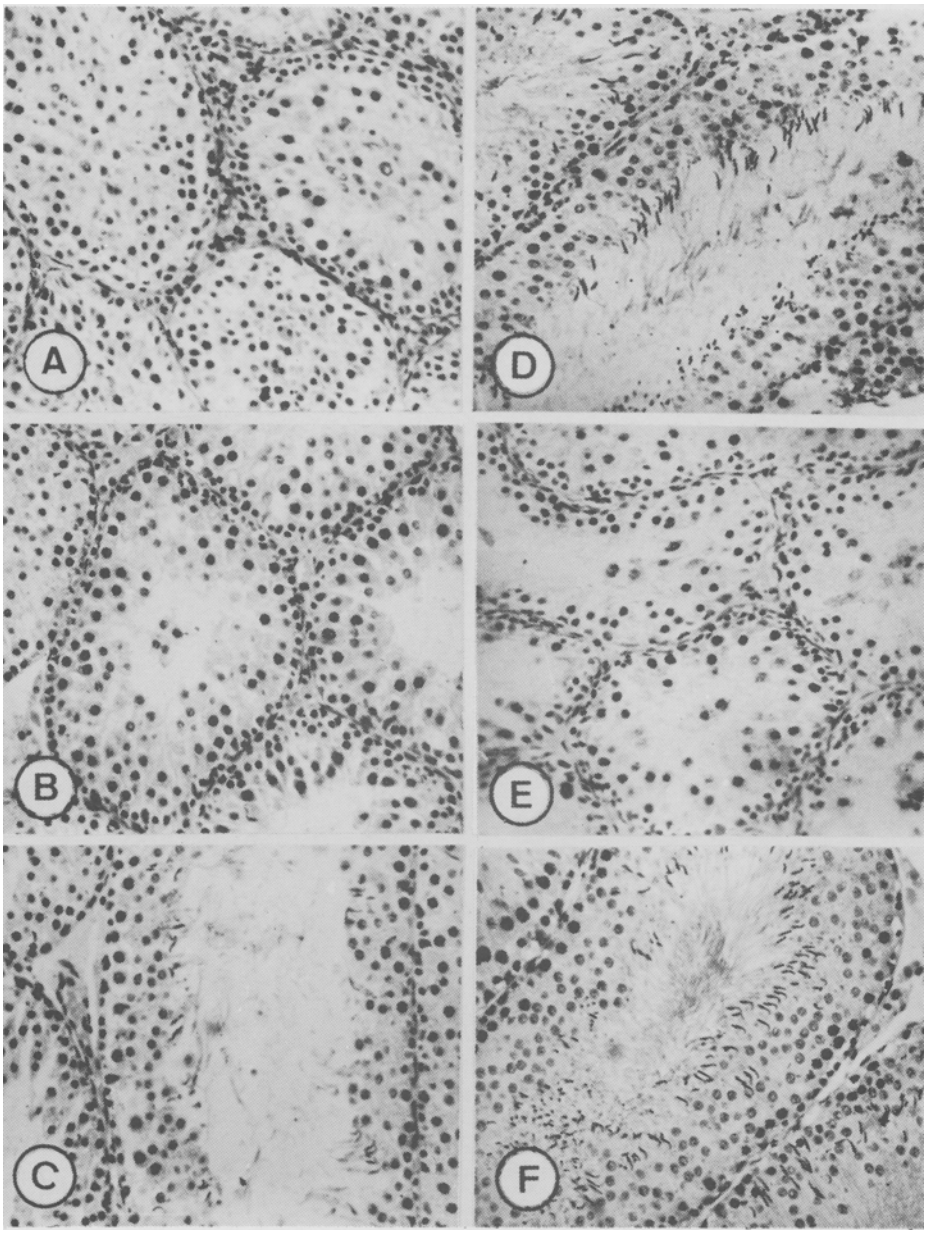

FIG. 2. Histology of the testes of rats feeding on sunflower seed oil: (A) 7 weeks of age: (D) 9 weeks of age; methyl palmitate: (B) 7 weeks of age, (E) 9 weeks of age; and fish oil: (C) 7 weeks of age (F) 9 weeks of age: hematoxylin-eosin (250 x).

epithelium. However, the effect is more pronounced at 9 weeks of age.

Animals fed the EFA-free diet showed, either at 7 or 9 weeks of age, a remarkable inhibition of spermatogenesis. In most of the animals, the maturation was stopped at the stage of primary spermatocyte; in others, early spermatids were also detected.

Rats fed sunflower seed oil may be considered EFA sufficient animals and normal from a dietary point of view. Testes of these rats, in general, showed an incomplete maturation at 7 weeks of age. However, at 9 weeks the majority of rats showed the full spermatogenic cell series, although the number of spermatozoa was low or moderate.

In the animals fed the fish oil, the maturation of germinal cells proceeded, in general, faster than in the other groups. A small or moderate number of spermatozoa was seen in about 50% of the animals 7 weeks of age. In others, maturation reached the early or late spermatid stage. In the oldest animals (9 weeks), spermatogenesis was normal in practically all the animals, and abundant spermatozoa were seen in the lumen of the tubules. No histological changes were detected in the interstitial tissue.

DISCUSSION

Results discussed in the preceding pages show that replacement of dietary linoleic acid by acids of the α-linolenic series caused a change in the fatty composition of testicular lipids. This change followed in some ways an EFA deficient pattern, but the loss of 22:5ω6 acid was compensated for by a deposition of 22:6ω3 in the same lipids. Therefore, the change led to a composition that is no longer typical of rats, but approaches the pattern in mouse or guinea pig in which 22:6ω3 and 22:5ω6 coexist (1).

Fish oil diet altered slightly Δ6 desaturation capacity of testicles in the same direction as EFA deficiency does. The small accumulation of 20:3ω9 and the decrease of 20:4ω6 in phospholipids evoked by the fish oil diet also resemble EFA deficiency. However, in spite of these changes, the body and testicular weights were not decreased, and the maturation of germinal cells of testes was normal or achieved even earlier than with the linoleic acid diet. Therefore, we suggest that the function of 22:5ω6 acid in testicles probably may be carried out by 22:6ω3 acid. Moreover, the typical composition of the fatty acids of rats testes published in the literature showing an abundance of 22:5ω6 acid and an absence of 22:6ω3 acid may be a consequence, at least partially, of the typical composition of the food of laboratory rats they generally receive. This diet is free or practically free of acids of the α-linolenate family.

The fact that some EFA deficient signs in the composition and enzyme activity are shown in the testes of rats fed fish oil does not necessarily contradict these results and conclusions since it is generally admitted that 22:5ω6 acid has a specific effect on the testes different from that of arachidonic acid.

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The Dietary Regulation of Stearyl Coenzyme A Desaturase Activity and Membrane Fluidity in the Rat Aorta

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ABSTRACT

Numerous studies have demonstrated that alterations in membrane composition or fluidity are often associated with alterations in the properties of membrane-bound enzymes. In order to obtain membranes of varying fluidity, rats were fed diets that were either fat-free or supplemented with 15% safflower oil, and two properties associated with aorta and liver microsomal membranes were selected for study: stearyl CoA desaturase activity, and fluidity as monitored by fatty acid composition and microviscosity (measured by fluorescence depolarization). If fluidity directly modulates desaturase activity, one would predict that a low fluidity would stimulate the desaturase activity. Ten times more desaturase activity is present in aorta microsomes from rats on a fat-free diet than in microsomes from rats on a safflower oil supplemented diet. However, on the fat-free diet, these aorta microsomes were more fluid than those of rats fed safflower oil supplemented diet. The fluidity of liver microsomal membranes was not altered in response to diet, despite significant changes in desaturase enzyme content. The contrasting evidence presented here suggests that no correlation exists between desaturase enzyme activity and membrane fluidity in the two tissues studies. We have demonstrated that the aorta has appreciable capacity to desaturate stearyl CoA and that dietary manipulation causes significant changes in aorta membrane fluidity that may be of sufficient magnitude to affect the overall metaboism of aorta cells.

INTRODUCTION

Consumption of diets containing polyunsaturated fatty acids is an acceptable form of therapy in the treatment of human atherosclerosis. Information concerning the enzymatic and compositional changes produced in the membranes of the aorta tissue by including polyunsaturated fatty acids in the human diet is scant, and animal model studies are a reliable and effective method of examining these effects. Our study was undertaken in order to compare some properties of the aorta microsomal membranes isolated from rats consuming either a polyunsaturated fatty acid supplemented diet or a fat-free diet. Since many studies on the influence of diet on liver microsomal membranes already exist, these membranes were also isolated for comparative studies. The microsomal membrane properties that we chose to examine were the following: stearyl CoA desaturase activity, the fatty acid composition of the membranes, and membrane microviscosity.

The stearyl CoA desaturase activity was selected since it may play a key role in the lipid metabolism of the aorta. This enzyme has been extensively studied in liver, and it has been shown to be a multicomponent membrane bound enzyme associated with the microsomal electron transport chain (1-3). The liver enzyme has been shown to have a lipid requirement for activity (4) and is under dietary regulation (5-9). The stearyl CoA desaturase has not been assayed directly in the aorta, but studies demonstrating [^{14}C]-acetate incorporation into oleic acid have been reported (10). The product of the desaturase reaction, oleyl CoA (11), has two probable fates in the aorta: esterification to cholesterol and incorporation into membrane phospholipids.

Two other membrane properties, fatty acid composition and membrane microviscosity, were selected in order to provide some information concerning the physical state of the aorta membranes in response to dietary challenges. Numerous studies have shown that alterations in membrane composition or membrane fluidity are often associated with alterations in the properties of membrane-bound enzymes. A recent review (12) has extended the documentation of this type of modulation to allosteric enzymes, particularly the Na+ - K+ ATPase. Membrane microviscostiy as measured by fluorescence depolarization with diphenylhexatriene has been shown to correlate well with direct thermodynamic measurements of artificial lipid bilayers (13), and this procedure appears to be useful in explaining many of the properties of natural membranes (14).

EXPERIMENTAL PROCEDURES

Animals

Male rats of the Wistar strain were used throughout these experiments. One group of rats consisted of mature males weighing 250-300 g, and they were maintained on the specified diets for up to 15 weeks. The rats were weighed regularly to ensure that adequate amounts of the diet were being consumed to maintain body weight. At each time period tested, two rats from each dietary group were sacrificed by decapitation, the blood collected with 0.2 ml of 0.1 M EDTA (pH 7.0), and pooled for each pair of animals. The liver and aorta tissues were likewise pooled from each pair of animals.

A second group of rats consisted of 50-60 g weanlings, and they were maintained on the test diets for up to 8 weeks and were processed as described above. The rats were weighed regularly, and growth charts were similar for the rats on the two diets. The weanling rats that were fed a fat-free diet had symptoms of essential fatty acid deficiency (fur texture changes) after 3 weeks of the diet. None of the adult rats tested showed these symptoms, even after 15 weeks of consuming a fat-free diet.

Diets

A standard modification of the Wooley and Sebrell fat-free test diet (15) was used. The diet contained 21.1% casein, 16.45% cellulose, 58.45% sucrose, 4.00% salt mixture, and 1 kg of total vitamin supplements per 100 lb of diet. All of the ingredients were obtained from Nutritional Biochemicals (Cleveland, OH), measured and mixed in our laboratory. The safflower oil supplemented diet contained 15% safflower oil added to the above fat-free diet.

Preparation of Microsomes

Liver microsomes: Livers, from two freshly decapitated rats, were pooled, washed in ice-cold 0.25 M sucrose, dried on filter paper, weighed, and homogenized in 5 vol of 0.25 M sucrose. Centrifugation of the homogenate at 20,000 x g for 10 min was followed by centrifugation at 100,000 x g for 60 min, and the pelleted microsomes from the second centrifugation were suspended in 0.25 M sucrose and used for assays.

Aorta microsomes: Rat aortas were dissected from the heart to the iliac bifurcation and washed in ice-cold 0.25 M sucrose, dried on filter paper, and weighed. The aortas from two rats were pooled, stripped of extraneous tissue, minced finely, and homogenized in 1 ml of 0.25 M sucrose. Centrifugation was performed as for liver microsomes. Approximately 1 mg of microsomal protein is isolated in this manner.

Protein

Protein was assayed by the Lowry method (16).

Enzyme Assays

Stearyl CoA desaturase activity was assayed using [1-^{14}C] stearyl CoA as has been previously described (11). Stearyl CoA used had a specific radioactivity of 3,900 cpm/nmole for the assay of the liver microsomes and 18,500 cpm/nmole for the assay of the aorta microsomes.

Cytochrome b_5 and stearyl CoA-stimulated cytochrome b_5 oxidase were assayed as previously described (9).

Fatty Acid Analysis

Fatty acid composition of total microsomal lipid and total plasma lipid (not merely nonesterified fatty acids) was determined by gas liquid chromatography as previously described (9).

Membrane Microviscosity

An aliquot of membrane preparation containing 100 μg of protein was diluted to 3.5 ml with 10 mM potassium phosphate buffer (pH 7.4), 2 μl of 1 mM 1,6-diphenylhexatriene (recrystallized, Puriss grade, Aldrich Chemical Company, Milwaukee, WI) in tetrahydrofuran was added, and the suspension mixed thoroughly by means of a vortex mixer. The sample was incubated at 37 C for 30 min and then transferred to a cuvette placed in a thermoregulated cuvette holder at 37 C in a Hitachi-Perkin Elmer spectrofluorimeter Model MPF-3. The sample was excited at 360 nm, and emitted light was collected at 430 nm. Fluorescence intensity and polarization were detected using polarizers oriented parallel and perpendicular to the direction of polarization of the excitation beam yielding $I_{\parallel}$ and $I_{\perp}$ (the fluorescence intensities). The contribution of light scattering to the intensity at 430 nm was less than 3%, and the values were corrected accordingly.

From the experimentally determined values of fluorescence, intensities $I_{\parallel}$ and $I_{\perp}$ values of the anisotropy (r) were calcualted. The microviscosity in the neighborhood of the probe molecules was then calculated from the Perrin equation:

$$r_o/r = 1 + C(r)\, T\tau/\eta$$

r_o is the limiting anisotropy of 1,6-diphenyl-

TABLE I

Influence of Diet on Stearyl CoA Desaturase Activity of Microsomes[a]

	Desaturase activity (nmole/min/mg)			
	Liver		Aorta	
Weeks on diet	Fat-free	Safflower	Fat-free	Safflower
Adult				
1	0.60	0.40	0.076	0.056
5	0.66	0.17	0.13	0.025
10	0.82	0.19	0.25	0.016
15	0.68	0.21	0.25	0.017
Weanling				
3	1.11	0.28	0.40	0.08
8	0.93	0.26	0.30	0.05

[a]Rats were fed special diets for the time periods indicated. Assays were performed as described in Materials and Methods. The numbers represent the mean values of four determinations.

hexatriene, given as 0.362 [Shinitzky and Barenholz (17)]. r is the measured anisotropy as defined by $r = I_{\parallel} - I_{\perp}/I_{\parallel} + 2T_{\perp}$. C(r) is a parameter related to the molecular shape of the fluorophore. A value of 8.6×10^5 poise deg^{-1} s^{-1} was reported by Shinitzky and Inbar (18). T is the absolute temperature. τ is the excited state lifetime and was estimated to be 6 nsec at 37 C from the data of Shinitzky and Barenholz (17) for 1,6-diphenylhexatriene dissolved in lecithin liposomes.

RESULTS

Adult rats were fed a fat-free diet or a diet supplemented with 15% safflower oil for up to 15 weeks, microsomes were prepared from aorta and liver tissue and assayed for stearyl CoA desaturase activity. No attempt to dissect the aorta into intima media cells and adventitia cells was made at this time, but the relatively small number of cells present in the adventitia portion of the aorta makes it unlikely that the adventitia contributes significant numbers of microsomes to this preparation. After 15 weeks of these two diets, tissues from the animals on a safflower oil supplemented diet had a much lower level of stearyl CoA desaturase activity than tissues from animals on a fat-free diet (Table I). A tenfold difference was observed in aorta microsomes and a threefold difference was observed in liver microsomes. Irrespective of diet the specific activity of the desaturase in the liver microsomes was considerably higher than the desaturase activity in the aorta microsomes. For example, on a fat-free diet, the desaturase activities found in liver and aorta microsomes were 0.68 and 0.25 nmole/min/mg, respectively. This difference is not altogether surprising as the content of endoplasmic reticulum in aorta is not as extensive as that of liver. Desturase activity of liver and aorta microsomes derived from weanling rats was likewise measured, since these animals had become essential fatty acid deficient. No significant difference between these results and those for adult rats that were not essential fatty acid deficient was detected, although the individual values attained for the enzyme activities of the preparations were somewhat different.

Although the subcellular localization of the desaturase in aorta has not as yet been established, the stearyl CoA desaturase of aorta microsomes is presumed to be associated with the microsomal electron transport chain, in a manner similar to the liver enzyme (19). Preliminary studies in our laboratory indicate that cytochrome b_5 is present in aorta microsomes in concentrations of ca. 0.02 nmoles of cytochrome b_5 per mg microsomal protein as compared to 0.09 nmoles per mg microsomal protein in the liver microsomes of these animals. A more realistic comparison is made of aorta and liver microsomal desaturase activity per nmole of cytochrome b_5, assuming that the latter protein is directly involved in the desaturase activity and is reliable as a endoplasmic reticulum marker protein. When this ratio is calculated using the above data for animals fed a fat-free diet, the ratios for aorta and liver are 12.5 and 7.6, respectively.

Further confirmation of the postulated role of cytochrome b_5 in stearyl CoA desaturation in aorta microsomes comes from an alternative assay procedure for desaturation (3). The spectrophotometric measurement of stearyl CoA-stimulated reoxidation of cytochrome b_5 (9) has been measured in both aorta and liver

TABLE II

Fatty Acid Composition of Tissues from Rats[a] Fed Special Diets

Tissue	Fatty Acid									
	16:0	16:1	18:0	18:1	18:2	20:3	20:4	22:6	Unknown	S/U ratio[b]
	(mole percent)									
Liver microsomes										
Fat-free diet	19.0	9.1	20.9	28.0	0.7	15.3	5.5	1.5	---	0.66
Safflower diet	13.4	0.4	22.6	3.9	21.0	---	34.2	0.9	3.5	0.56
Aorta microsome										
Fat-free diet	25.0	6.8	18.0	30.3	---	12.1	7.8	---	---	0.75
Safflower diet	28.3	2.9	27.1	15.3	11.4	---	15.0	---	---	1.20
Plasma										
Fat-free diet	24.0	9.1	19.2	29.3	---	17.4	---	---	---	0.77
Safflower diet	17.3	0.3	22.4	8.3	32.0	---	19.7	---	---	0.65
Adipose tissue										
Fat-free diet	25.0	12.0	3.6	58.7	---	---	---	---	0.4	0.40
Safflower diet	19.0	3.7	2.6	18.9	44.0	---	---	---	11.3	0.32

[a]Tissues from weanling rats fed special diet for 8 weeks were saponified as described in Materials and Methods. The methyl esters of the resultant fatty acids were prepared and separated by means of gasliquid chromatography. Components not detectable would constitute less than 0.1% of the total fatty acid present.

[b]S/U ratio is calculated by dividing the total moles of saturated fatty acid by the total moles of unsaturated fatty acid.

microsomes. The rate of reoxidation of cytochrome b_5 in the presence of stearyl CoA is ca. 0.15 nmoles/min/mg protein at 37 C in aorta microsomes as compared to 0.65 nmoles/min/mg protein in liver microsomes. These values are derived from the liver and aorta of rats fed a fat-free diet for 15 weeks, and they compare well with desaturase activity determined by the radioactive procedure (Table I).

These data indicate that the ratio of units of desturase per nmole of cytochrome b_5 is high in the aorta preparations and that the ability of the desaturase to accept electrons from cytochrome b_5 is at least as great in the aorta microsomes as in liver microsomes. This suggests that the endoplasmic reticulum of aorta has a very high capacity for stearyl CoA desturation and that this enzyme, which has not been documented extensively in other tissues (20,21), is not restricted to hepatic tissue.

The changes in stearyl CoA desaturase activity in aorta and liver microsomes seen in both adult and weanling rats is accompanied by changes in the fatty acid composition of these microsomes. Microsomes from weanling animals fed a safflower oil supplemented diet for 5 weeks contain high concentrations of linoleic acid and arachidonic acid (Table II). Microsomes from animals fed a fat-free diet for 5 weeks have high concentrations of oleic and palmitoleic acid, a dwindling concentration of linoleic acid, and a high concentration of eicosatrienoic acid. These fatty acid compositions are similar to those of others for the liver microsomes of rats fed similar diets (22). A quantitative comparison of the moles of fatty acid present in liver microsomal membranes as saturated fatty acid or as moles of unsaturated fatty acid can be made and a ratio can be calculated as in Table II. As can be seen, the fatty acids of liver microsomal membranes from both sets of animals are present in a ratio of saturated to unsaturated (S/U ratio) that is similar despite the qualitative difference in fatty acid content. This is in contrast to the aorta microsomal membranes where the S/U ratio varied in response to the diet consumed by the animal. In aorta microsomes from animals fed a fat-free diet, the ratio is 0.75, but on a safflower oil supplemented diet this ratio is 1.25. This indicates that more saturated fatty acids are present in these membranes following the feeding of a safflower oil supplemented diet. In order to determine if the higher S/U ratio in aorta microsomes from safflower oil fed rats is a result of an influx of fatty acids from the plasma, with a similar S/U ratio, this ratio was calculated for the total plasma fatty acids of the two sets of rats. No significant difference in the S/U ratio of total plasma fatty acids was detected (Table II). The fatty acid composition of weanling rat adipose tissue indicate that the weanling rats fed a fat-free diet were essential fatty acid deficient.

The hydrophobic probe 1,6-diphenylhexatriene was used to measure the membrane microviscosity of the microsomal fractions from liver and aorta tissue. Numerous studies

TABLE III

Influence of Diet on the Microviscosity of Microsomes[a]

	Microviscosity (poise) (37 C)			
	Liver		Aorta	
Weeks on diet	Fat-free	Safflower	Fat-free	Safflower
Adult				
1	0.79	0.89	0.62	0.62
5	0.95	0.93	0.87	1.24
10	0.68	0.56	0.93	1.33
15	0.77	0.64	0.94	1.58
Weanling				
5	0.81	0.70	1.0	1.75
8	0.66	0.70	1.1	1.83

[a]Rats were fed special diets for the time period indicates. Microviscosity was assayed as described in Materials and Methods. The numbers represent the mean values of three determinations.

(13,14,17,23) have demonstrated that analyzing the fluorescence depolarization properties of this probe embedded in membranes can lead to a quantitative evaluation of the fluid state of the membrane lipid bilayer. In Table III, the values for the microviscosity at 37 C of liver and aorta microsomal membrane fractions from animals fed the two sets of diets are reported. The values for microviscosity for liver microsomal membranes do not vary significantly in response to the diets tested despite the qualitative change in fatty acid composition. This is in agreement with the value for the S/U ratio for the liver microsomes which suggests that the saturated and unsaturated fatty acids are adjusted so that they are in a constant ratio despite qualitative shifts caused by dietary challenges. Microviscosity values for aorta microsomes are also reported in Table III. The microviscosity of aorta microsomes is considerably affected by the diet consumed by the animal. The higher microviscosity value for the aorta from animals fed polyunsaturated fatty acid diets is unexpected since this diet is more "fluid" and would be predicted to produce more "fluid" membranes. However, these data are in agreement with the S/U ratio calculated in Table II from the fatty acid composition of these aorta microsomes. This ratio indicates more saturated fatty acids are present in these aorta membranes than in the aorta membranes of rats fed a fat-free diet. The values for microviscosity of the aorta membranes for rats fed a fat-free diet vary somewhat with time but make no significant change in the time periods tested. The microviscosity of plasma samples from rats fed the test diets indicated that no change in the microviscosity values of this tissue occurs in response to diets tested.

DISCUSSION

Fatty acid synthesis in the aorta has been observed previously, and an extensive review of this subject has been made (24). A major factor affecting the regulation of fatty acid synthesis in the aorta is cholesterol feeding, which results in a greatly acceleratted rate of fatty acid synthesis. St. Clair et al. (10) found that in atherosclerotic aortas all lipid classes were increased but that synthesis of oleic acid and its esterification to cholesterol was stimulated to the greatest extent. The enzyme responsible for oleic acid synthesis from stearic acid is the stearyl CoA desaturase, and the regulation and characterization of this enzyme in aorta tissue has not been previously investigated. The regulation of the biosynthesis of oleic acid is not only a factor in cholesterol metabolism but is also important in the regulation of the fatty acid composition of the membranes synthesized by the organism.

Several membrane-bound enzymes have been shown to be modulated by alterations in the physical properties of the membrane, and a relationship between membrane fluidity and the regulation of membrane-bound enzymes as a general biological phenomena has been recently reviewed (12). The stearyl CoA desaturase is a multicomponent membrane-bound enzyme, and it has been demonstrated that the three separate proteins that constitute the complete desaturase complex must be present in a membrane of sufficient fluidity in order for protein-protein interactions to take place (25). The latter studies were carried out in vitro using purified liposomes and purified proteins.

The advent of techniques to measure fluidity, or its reciprocal viscosity, has enabled correlations between fluidity of naturally occurring membranes to be made with both

activity and chemical composition of these membranes. An estimation of the fluidity of microsomal membranes from rat liver and aorta was made by direct measurement of microviscosity and fatty acid composition. In the liver microsomes, dietary-induced changes in desaturase activity are not associated with fluctuations in membrane fluidity as the values for the microviscosity of these membranes are remarkably constant throughout the course of the experiment. Maintenance of a constant membrane fluidity, in order to maintain optimal membrane function, appears to be accomplished in this tissue despite the vastly different diets consumed by the whole animal. In the aorta microsomes, membrane fluidity does not appear to be maintained in a constant fashion, as the microviscosity of the aorta microsomes increases under safflower oil feeding conditions. If the desaturase activity is controlled by the fluidity of the microsomal membrane, the desaturase activity would be predicted to be stimulated under conditions of high microviscosity in order to produce more double bonds to return to the original microviscosity. The desaturase activity, however, is inhibited in these microsomes. Future experiments using purified preparations of microsomal membranes will be necessary in order to determine that membrane fluidity changes observed in the aorta are localized in the endoplasmic reticulum and not in the plasma membrane fraction alone. If these experiments confirm our preliminary observations that a high microviscosity does not stimulate desaturase activity, then a reevaluation of the relationship of membrane fluidity and enzymic regulation may be necessary. It is also possible that desaturase activity may be controlled by the fluidity of the microenvironment in the region of the desaturase enzyme and not by the overall fluidity of the microsomal membrane.

We have presented evidence that a less fluid membrane fraction is present in aorta from rats fed a polyunsaturated fatty acid diet, but we have not addressed the crucial question of defining completely the chemical changes that have brought about this fluidity change. There is a shift to a more saturated fatty acid content in these membranes, and our enzymatic studies indicate that oleic acid is not readily synthesized in these membranes. Another significant contribution to the fluidity of membranes is the cholesterol content of the membrane. We have no direct measurement of this parameter as yet due to the small amount of membrane material available and the necessity for developing an assay in the 1-5 μg range (This is possible by gas liquid chromatography). Some implications from the fatty acid data may allow us to speculate that cholesterol levels are elevated in the membranes of aorta from polyunsaturated fatty acid fed rats. Cholesteryl oleate formation cannot proceed normally in these rats since oleate production by the desaturase is inhibited, hence the cholesterol may accumulate in the membranes of the aorta. We have no data at this time on the synthesis of cholesteryl oleate in these membranes, but future experiments are planned to measure this process.

Prior to this report, no correlations had been made between dietary lipid and aortal membrane fluidity. The relevance of these studies to human nutrition is obvious, especially with the wide spread use of diets rich in polyunsaturated fatty acids. Although we have no way of knowing whether alterations of aortal membrane fluidity can give rise to pathological states, or even whether a high fluidity is more beneficial than a low fluidity in a particular membrane, we feel that the fluidity differences that we observe need to be investigated further.

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Glyceride Structure Variation in Soybean Varieties: I. Stereospecific Analysis[1]

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ABSTRACT

Stereospecific analysis of soybeans and related species showed that there was little palmitic or stearic acid on the *sn*-2-position, and the *sn*-1-position is consistently richer in palmitic, stearic, and linolenic acids than the *sn*-3-position. The *sn*-3-position is enriched in oleic acid and the *sn*-2-position with linoleic. Plots of the percentage of fatty acids on the glycerol positions vs. the percentage in the whole oil revealed a soybean variety that had a deviant distribution that is probably genetically controlled.

INTRODUCTION

The glyceride structure of fats and oils may influence their stability towards oxidation (1-3). Weber and Alexander (4) have reported genetic differences in the glyceride structure of corn. The glyceride structure of soybean oil has been examined during maturation by silver ion chromatography (5), by pancreatic lipase analysis (5-9), and by sterospecific analysis (10), but only a limited number of varieties have been studied for glyceride composition by lipase and silver ion chromatography (11). This paper reports the variation in glyceride structure determined by stereospecific analysis of a number of soybean varieties and closely related species selected to embrace a wide range of fatty acid compositions.

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TABLE I

Average Differences between the Percentage of Fatty Acids Determined on the *sn*-3-Position by Two Methods of Calculation and the Standard Deviation of the Differences

Fatty acid	Ave. diff.	S.D.
16:0+18:0	0.45	0.26
18:1	1.63	0.71
18:2	1.24	0.47
18:3	0.37	0.20

MATERIALS AND METHODS

Beans were obtained from the Agronomy Department of Iowa State University. These included soybeans, *Glycine max* (L.) Merr., which were commercial cultivars, lines produced in breeding programs to alter fatty acid composition (FA), and plant introductions (PI). In addition, one variety each of *Glycine soya* and *Glycine gracilis* were examined.

The soybeans (50 g) were ground in a Wiley mill and extracted with hexane. Triglycerides were isolated from the lipid extract by thin layer chromatography (12). Lipase hydrolysis was done according to Luddy et al. (13), and sterospecific analysis according to Christie and Moore (14). For fatty acid analyses, the glycerides were transesterified with 0.1 M sodium methoxide in methanol, and the methyl esters were separated on a Beckman GC5 instrument fitted with a 1.8 m x 3.3 mm column of 15% EGSSX on Chromosorb W and a hydrogen flame detector.

Data plots were fitted with straight lines by linear regression (15). Data for PI 68-788 and *G. soya* were omitted in the regression.

RESULTS

The stereospecific analysis yields two ways of calculating the composition of the *sn*-3-position of the triglycerides. The *sn*-3-position can be obtained by subtracting the composition of the *sn*-1- and *sn*-2-position from the composition of the whole triglycerides or by subtracting the composition of the *sn*-2-position from that of the *sn*-2,3-diacyl-phosphatides that are left unhydrolyzed by snake venom. The former is regarded as the more accurate, but the agreement of these two values is a general check on the reliability of the method (14). The average difference in the two values and the standard deviation of the differences for each fatty acid are given in Table I.

Amsoy and Corsoy cultivars grown in three separate years were analyzed to obtain an estimate of the size of seasonal effects of the positional distribution. These results are shown along with others in Table II. There are small year-to-year differences in the percentages of the fatty acids on the three positions of the glycerol, but these can mostly be attributed to the change in fatty acid composition from year

TABLE II

Stereospecifc Analysis of Soybeans and Closely Related Species
(Results are in mol percent)

Variety	Compound or position	16:0	18:0	18:1	18:2	18:3
Amsoy 73	TG	11.14	3.95	21.84	53.92	9.15
	1	20.72	6.78	19.66	42.89	9.94
	2	0.69	---	19.03	71.74	8.54
	3	11.85	5.00	26.83	47.13	8.97
Amsoy 74	TG	10.99	3.65	23.73	52.56	9.07
	1	21.11	6.58	20.71	42.44	10.16
	2	0.60	0.11	21.16	69.15	8.99
	3	12.15	4.22	29.33	46.07	8.07
Amsoy 75	TG	11.53	3.49	25.32	51.86	7.80
	1	21.34	5.98	22.46	41.29	8.93
	2	0.63	---	23.54	68.39	7.44
	3	12.44	4.45	29.96	45.89	7.04
Corsoy 73	TG	11.63	3.68	23.19	52.81	8.68
	1	21.15	5.80	18.99	44.38	9.68
	2	1.03	0.27	19.76	70.63	8.32
	3	12.55	4.93	30.83	43.44	8.04
Corsoy 74	TG	11.54	3.71	22.99	53.43	8.33
	1	20.99	6.34	18.96	43.58	10.09
	2	0.61	---	19.29	72.31	7.79
	3	12.84	4.74	30.73	44.39	7.09
Corsoy 75	TG	12.13	3.34	23.27	53.89	7.37
	1	21.93	5.87	19.79	43.64	8.78
	2	0.60	---	21.64	70.94	6.82
	3	13.69	4.09	28.36	47.09	6.54
Wells	TG	11.07	3.78	20.09	57.07	7.98
	1	19.52	6.30	19.24	44.58	10.37
	2	1.02	0.30	19.31	72.68	6.86
	3	12.54	4.70	21.89	53.97	6.71
PI 68-543	TG	12.30	4.21	22.92	53.73	6.85
	1	21.38	6.92	20.33	43.09	8.28
	2	0.99	0.30	22.75	69.82	6.14
	3	14.35	5.34	25.68	48.27	6.13
PI 70-091	TG	11.94	3.98	24.32	53.00	6.75
	1	21.99	6.85	20.89	42.87	7.40
	2	0.85	0.21	23.90	68.77	6.28
	3	12.82	4.83	28.17	47.37	6.58
PI 68-788	TG	13.32	4.15	28.59	47.18	6.77
	1	21.52	6.20	22.67	42.10	7.51
	2	0.87	---	24.74	67.69	6.71
	3	17.38	6.19	38.26	31.76	6.08
FA 3171-2	TG	9.21	3.00	51.34	31.89	4.56
	1	16.75	4.56	46.47	27.39	4.82
	2	0.60	0.10	52.74	41.78	4.77
	3	10.16	4.30	54.79	26.51	4.09
FA 3043-2	TG	10.98	3.35	39.77	40.03	5.86
	1	19.19	5.48	35.75	32.99	6.59
	2	0.76	0.18	39.92	53.52	5.62
	3	12.85	4.36	43.66	33.57	5.38
FA 3017-2	TG	9.14	2.79	53.00	30.21	4.86
	1	16.16	5.00	48.24	25.31	5.28
	2	0.84	0.18	54.66	39.39	4.93
	3	10.33	3.16	56.11	25.91	4.35
FACO 2-8203	TG	11.49	4.17	33.57	44.85	5.91
	1	19.64	6.45	29.86	37.38	6.66
	2	0.99	---	33.86	59.72	5.43
	3	13.70	6.02	37.00	37.45	5.63
FACO 2-8256	TG	10.24	3.84	44.93	35.69	5.29
	1	16.95	5.82	41.34	30.09	5.79
	2	0.88	---	48.86	45.71	4.54
	3	12.76	5.65	44.59	31.28	5.55
PI 65-549	TG	13.38	3.28	12.64	55.34	15.36
(*G. soya*)	1	23.84	5.61	11.28	43.39	15.87
	2	0.69	---	10.67	73.49	15.16
	3	15.39	4.17	15.96	49.15	15.05
PI 135-589	TG	14.35	3.56	15.33	55.17	11.58
(*G. gracilis*)	1	25.77	5.73	13.25	41.38	13.88
	2	1.12	0.22	13.72	75.11	9.83
	3	15.92	4.68	19.00	49.06	11.03

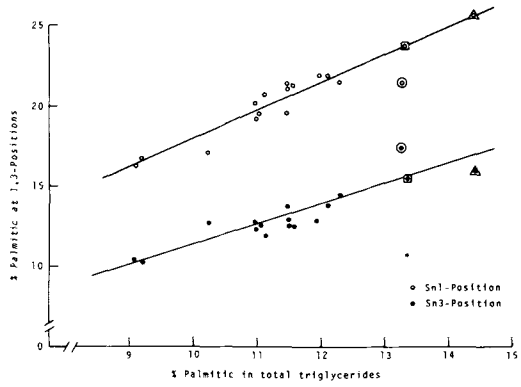

FIG. 1. Percentage of palmitic acid on the *sn*-1 and *sn*-3-positions of glycerol vs. the percentage of palmitic acid in the triglyceride. (○) PI 68-788, (□) *G. soya*, (△) *G. gracilis.*

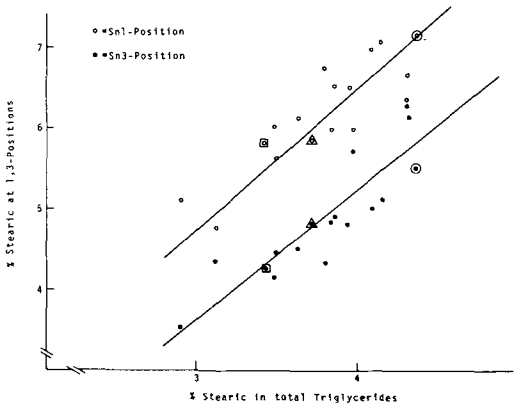

FIG. 2. Percentage of stearic acid on the *sn*-1- and *sn*-3-positions of glycerol vs. the percentage of stearic acid in the triglyceride. (○) PI 68-788, (□) *G. soya*, (△) *G. gracilis.*

to year. The good agreement among the cultivars grown in different years is a further indication of the reporducibility of the analyses.

The results of Table II are generally in close agreement with those previously reported on soybean oil which were determined by pancreatic lipase (5-9,11) and a single stereospecific analysis (10). There is very little stearic or palmitic acid on the *sn*-2-position, and the *sn*-1-position is consistently richer in palmitic, stearic, and linolenic acids than is the *sn*-3-position. The *sn*-3-position is richer in oleic acid than is the *sn*-1-position, and the *sn*-2-position is enriched in linoleic and oleic acid.

If the percentage of a fatty acid at one of the positions of the glycerol is plotted vs. the percentage of that fatty acid in the whole fat (Fig. 1-5), one generally gets a straight line. This has been reported for other fats and oils

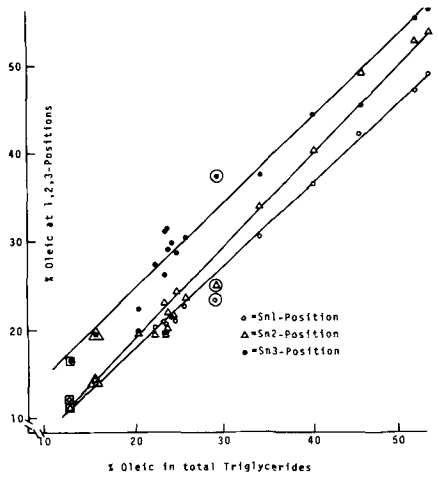

FIG. 3. Percentage of oleic acid on the *sn*-1-, *sn*-2-, and *sn*-3-positions of glycerol vs. the percentage of oleic acid in the triglyceride. (○) PI 68-788, (□) *G. soya*, (△) *G. gracilis.*

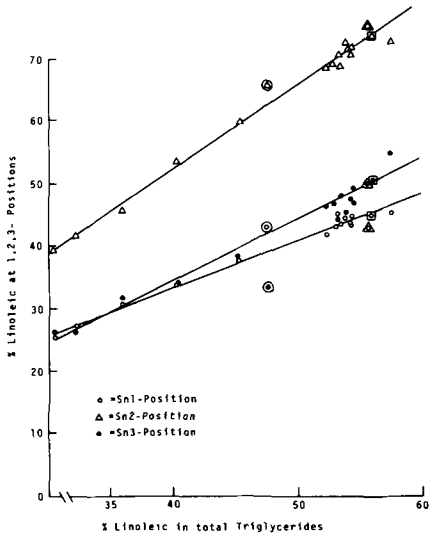

FIG. 4. Percentage of linoleic acid on the *sn*-1, *sn*-2-, and *sn*-3-positions of glycerol vs. the percentage of linoleic acid in the triglyceride. (○) PI 68-788, (□) *G. soya*, (△) *G. gracilis.*

(12). The data in Table II were fitted by linear regression, and the slopes and intercepts found are given in Table III. The fits were highly significant, and most of the variance was accounted for by the linear regression. The linearity of these plots indicates that the mechanism responsible for the placement of the fatty acids on the three positions retains its specificity over the range of compositions investigated. The

slopes of the lines can range from 3 down. Slopes of 1 would be obtained for all instances if the distribution were random, and the slopes of all three positions for an acid should average 1. Slopes higher than 1 indicate that the placement of the fatty acids were favored on that position, and the higher it is, the more the placement is favored. The intercepts would be 0 if the plots could be extrapolated to zero. This is not found in Table III, but the algebraic sum of the intercepts is near 0 as would be required for a good fit over the range examined.

These plots are helpful in looking for genetic variation in the mechanism of distribution of the fatty acids on the positions of glycerol. If points for a fatty acid fall away from the linear plots, some change in mechanism of placement is indicated. Examination of the data indicates that points for PI 68-788 fell well off the plots for linoleic acid and were among the most deviant points on the oleic acid plots. This variety seems to have less linoleic acid on the *sn*-3-position and more on the *sn*-1- and 2-positions than would be expected from its fatty acid composition. We believe that this deviation is genetically controlled. Likewise, the values for PI 65-549, the *G. soya* variety tested, fall off the plots for linolenic acid. In this species, the linolenic acid is more evenly distibuted on the three positions of the glycerol.

The work of Raghuveer and Hammond (1) suggested that glycerides with unsaturated fatty acids on the *sn*-2-positions and saturated fatty acids on the *sn*-1- and 3-positions should be more stable to autoxidation. Rates of autoxidation are believed to increase with the number of double bonds in fatty acids (16-17). This suggests that the most stable oil would be one in which the linolenic and linoleic acids are concentrated on the *sn*-2-position. The two deviants found are in the right direction, but it is not clear if the deviations are enough to have a significant effect on oil stability. *G. Soya*, unfortunately, is very rich in linolenic acid. It will cross with *G. max*, and *G. gracilis* is thought to be such a cross. The distribution of

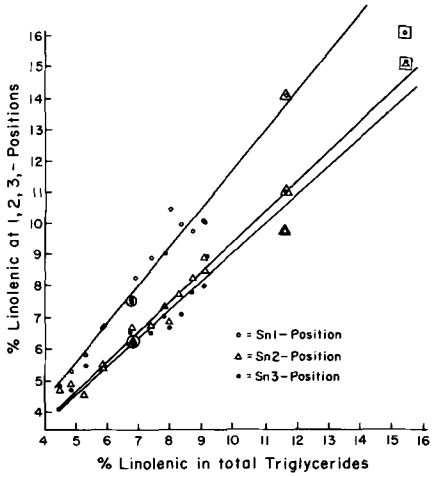

FIG. 5. Percentage of linolenic acid on the *sn*-1-, *sn*-2-, and *sn*-3-positions of glycerol vs. the percentage of linolenic acid in the triglyceride. (○) PI 68-788, (□) *G. soya*, (△) *G. gracilis*.

G. gracilis seems to follow the *G. max* distribution in the instance tested.

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TABLE III

The Slopes and Intercepts of the Linear Regression of the Percentage of a Fatty Acid at a Particular *sn*-Position vs. the Percentage of that Fatty Acid in the Whole Fat

	16:0		18:0		18:1		18:2		18:3	
Position	Slope	Inter	Slope	Inter	Slope	Inter	Slope	Inter	Slope	Inter
1	1.87	-0.86	1.49	0.61	0.94	-1.41	0.72	4.21	1.24	-0.63
2	—	—	—	—	1.14	-4.78	1.34	-0.84	0.84	0.67
3	1.05	0.86	1.48	-0.68	0.93	6.23	0.94	-3.45	0.92	0.00
Ave.	0.97		0.99		1.00		1.00		1.00	
Sum		0.00		-0.07		-0.04		-0.08		0.04

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Glyceride Structure Variation in Soybean Varieties: II. Silver Ion Chromatographic Analysis[1,2]

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ABSTRACT

The glyceride structures of 20 soybean varieties were determined by silver ion chromatography. Plots of the percentage of each triglyceride vs. the percentage of its constituent fatty acids revealed no genetic variants that had not been found previously by stereospecific analysis. The results deviated significantly from the amounts predicted by the 1-random-2-random-3-random hypothesis of glyceride distribution. This deviation can be explained by a noncoincidence of the peak biosynthesis of oleic and linoleic acids during maturation of the beans.

INTRODUCTION

The glyceride structure of fats and oils may influence their stability to autoxidation (1-3). In a companion paper (4), we reported stereospecific analyses of a number of soybean varieties and the discovery of two possible genetic variants from the general pattern. Weber and Alexander (5) have reported a genetic variant in the glyceride structure of corn. The corn variant also involved distribution of the fatty acids on the three glycerol positions. Besides stereospecific analyses (4,6) the glyceride structure of soybean oil has been examined by pancreatic lipase (7-11) and by silver ion chromatography during maturation (11) and for a limited number of varieties (12). This paper reports the glyceride structure variation by silver ion chromatography in a number of soybean varieties selected to embrace a large range of fatty acid compositions. The results are compared with those predicted by the 1-random-2-random-3-random hypothesis (8).

MATERIAL AND METHODS

Soybeans (*Glycine max* var. Merr) obtained from the Agronomy Department of Iowa State University were extracted, and the triglycerides were isolated as described previously (4). Silver ion chromatography was done as described by Roehm and Privett (11) using 2.5% methanol and chloroform as the developing solvent. This single system was sufficient to resolve the glycerides in mature beans. The plates were activated at 190 C, and the bands were eluted with ether-methanol with the aid of NaCl solution as recommended by Litchfield (13). Preparation of methyl esters and gas chromatography were as previously described (4). Methyl hepadecanoate was added to the silica gel during the extraction of the bands to serve as an internal standard.

A computer program was devised to calculate the glyceride composition from the fatty acid analysis of the bands recovered from the silver ion plates. The possible triglyceride species in each band of the silver ion plate were specified, and the computer tested appropriate equations until the set was found that summed correctly with no negative values. This set was used for the calculation. In a few instances, all possible sets yielded one or more negative values. In these instances, the set giving the smallest negative value was used. The negative value was always less than 1% of the glyceride in the band. A computer program was also devised to calculated the amounts of the triglycerides predicted by the 1-random-2-random-3-random theory and the stereospecific data obtained previously for some of the samples (4). The amounts predicted from

TABLE I

Average Differences in the Percentage of Triglycerides Determined by Silver Ion Chromatography and the Standard Deviation of Individual Determinations

	Ave. Diff.	S		Ave. Diff.	S
S_2M	.23	.17	SMT	.36	.15
M_2S	.28	.25	M_2T	.33	.30
S_2D	.33	.38	D_3	.39	.67
M_3	.25	.23	SDT	.21	.18
SMD	.21	.14	MDT	.27	.11
M_2D	.31	.19	D_2T	.84	.42
D_2S	.60	.27	T_2D	.31	.19
D_2M	.50	.27			

[1]Journal Paper No. J-8840 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA. Project No. 2143.

[2]Abbreviations used: S = saturated acyl group on a triglyceride, 18:0 + 16:0. M = monounsaturated acyl group, 18:1. D = diunsaturated acyl group, 18:2. T = triunsaturated acyl group, 18:3.

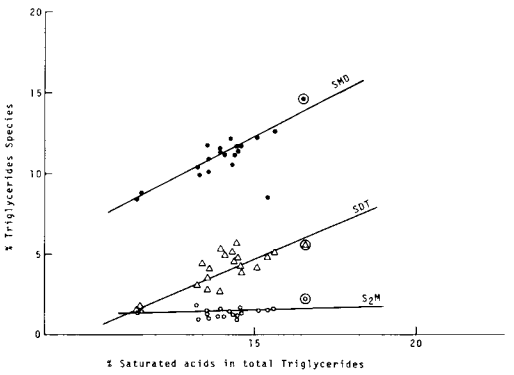

FIG. 1. Percentage of triglyceride species vs. percentage of saturated fatty acids in soybeans. ○, PI 68-788.

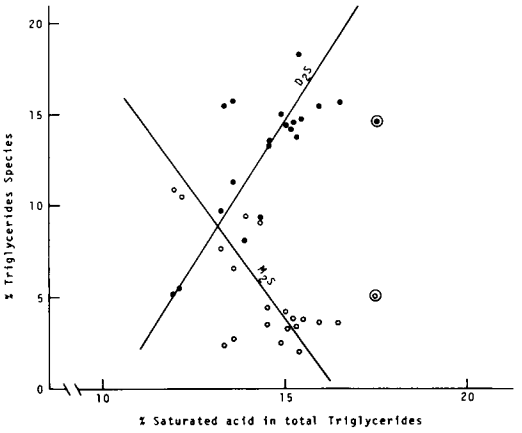

FIG. 2. Percentage of triglyceride species vs. percentage of saturated fatty acids in soybeans. ○, PI 68-788.

the theory were summed into the groups determined by silver ion chromatography for comparison.

RESULTS AND DISCUSSION

To check the reproducibility of the method, duplicate analyses were made on four commercial cultivars. Table I shows the average difference in duplicates for the various species of triglyceride and the standard deviation of individual analyses. Recovery of the triglycerides applied to the silver ion plates generally exceeded 96% based on the internal standard. Another check on the reliability of the method is the agreement between the fatty acid composition of the original triglycerides and the composition calculated from the proportions of the triglyceride species that were measured. Such a comparison is shown for several of the

TABLE II

Comparison of the Fatty Acid Composition of Some Soybean Varieties with that Calculated from the Silver Ion Fractions

Fatty acid	Williams		Clark		Steele		8882		8698	
	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated
16:0 + 18:0	14.26[a]	14.38	16.13	16.36	15.46	15.84	14.65	15.03	14.36	14.50
18:1	21.95	21.82	18.16	17.94	24.43	24.56	43.21	42.99	33.47	33.29
18:2	47.32	56.92	57.79	58.01	52.99	52.23	37.06	37.51	46.01	46.51
18:3	6.46	6.95	7.93	7.69	7.12	7.35	5.09	4.48	6.16	5.67

[a]Results are in mol percent.

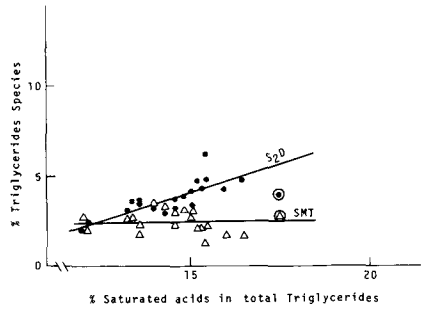

FIG. 3. Percentage of triglyceride species vs. percentage of saturated fatty acids in soybeans. O, PI 68-788.

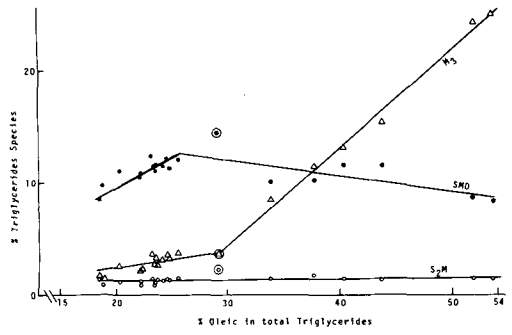

FIG. 4. Percentage of triglyceride species vs. percentage of oleic acid in soybeans. O, PI 68-788.

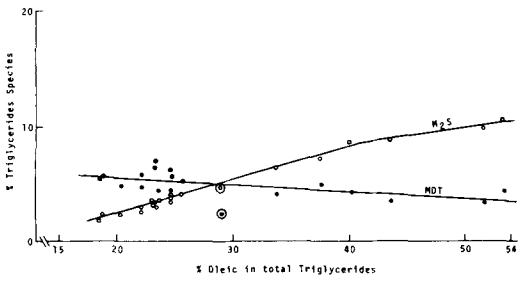

FIG. 5. Percentage of triglyceride species vs. percentage of oleic acid in soybeans. O, PI 68-788.

samples in Table II. These data indicate that the method is of satisfactory accuracy.

Table III shows the amounts of the triglyceride species measured for 13 selections. To test the variation with growing conditions, Amsoy and Corsoy samples grown in 1973, 1974, and 1975 were compared. The small differences in glyceride species can be attributed primarily to small variations in fatty acid composition.

The amounts of various triglyceride species given in Tables III plus data for seven additional

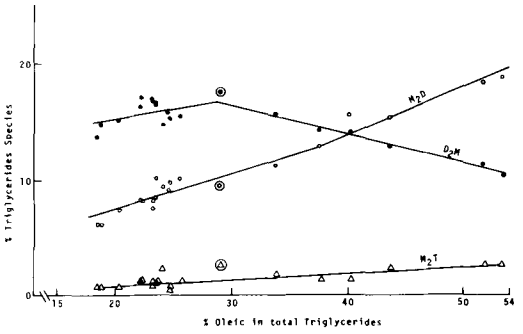

FIG. 6. Percentage of triglyceride species vs. percentage of oleic acid in soybeans. O, PI 68-788.

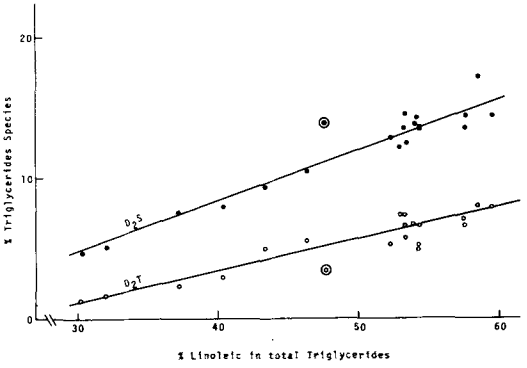

FIG. 7. Percentage of triglyceride species vs. percentage of linoleic acids in soybeans. O, PI 68-788.

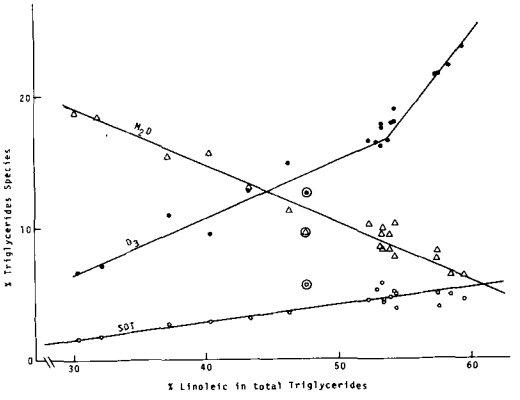

FIG. 8. Percentage of triglyceride species vs. percentage of linoleic acids in soybeans. O, PI 68-788.

varieties are plotted vs. the amounts of their constituent acids in the whole triglyceride in Figures 1 through 11. The point representing PI 68-788, which seemed to have a deviant distribution of palmitate, oleate, and linoleate on the three glycerol positions (4), is marked with a

TABLE III (Below and Opposite)

Triglycerides Found by Silver Ion Chromatography and Those Calculated by 1-random-2-random-3-random Theory

	Amsoy 73		Amsoy 74		Amsoy 75		Corsoy 73		Corsoy 74		Corsoy 75	
Species	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated
S_3	—	0.03[c]	—	0.03	—	0.03	—	0.06	—	0.03	—	0.03
S_2M	1.21	0.97	1.26	1.01	1.65	1.18	1.26	1.09	1.01	1.01	1.29	1.15
M_2S	3.34	2.08	3.63	2.42	4.19	2.87	3.35	2.38	3.89	2.31	3.77	2.51
S_2D	3.39	3.51	3.60	3.19	3.94	3.32	4.29	3.62	4.71	3.64	3.78	3.68
M_3	1.89	1.00	3.84	1.28	4.17	1.58	2.47	1.16	2.42	1.13	3.45	1.21
SMD	11.08	11.70	10.53	12.02	11.51	12.98	11.18	12.39	10.99	12.48	11.09	12.88
M_2D	7.75	7.74	8.79	8.85	9.48	9.94	8.31	8.47	8.07	8.43	9.55	8.68
D_2S	14.31	14.69	13.72	13.49	14.22	13.53	13.95	14.06	14.55	14.49	14.87	14.98
S_2T	—	0.43	—	0.42	—	0.37	—	0.45	—	0.40	—	0.36
D_2M	16.03	18.75	14.56	19.34	15.28	19.97	15.94	19.29	16.27	19.51	16.58	19.83
SMT	2.96	1.74	2.19	1.86	2.60	1.73	2.03	1.79	2.16	1.67	1.93	1.54
M_2T	1.50	1.29	2.53	1.53	1.49	1.50	1.31	1.38	1.04	1.31	1.51	1.20
D_3	17.25	14.50	15.75	13.52	15.83	12.96	16.01	13.61	16.14	13.99	18.36	14.57
SDT	5.36	4.78	5.51	4.44	4.72	3.86	6.08	4.47	5.03	4.29	4.06	3.88
MDT	5.49	6.58	5.71	6.91	4.99	6.25	6.71	6.55	6.12	6.38	4.22	5.67
D_2T	6.48	7.85	7.28	7.36	5.12	6.20	6.57	7.09	6.69	6.99	4.95	6.36
T_2S	—	0.36	—	0.35	—	0.26	—	0.33	—	0.29	—	0.24
T_2M	—	0.54	—	0.59	—	0.46	—	0.53	—	0.48	—	0.38
T_2D	1.97	1.37	1.07	1.29	0.84	0.95	0.54	1.19	0.89	1.11	0.61	0.88
T_3	—	0.08	—	0.07	—	0.05	—	0.06	—	0.06	—	0.04

[a]TG, triglyceride; S, saturated; M, monoenoic; D, dienoic; T, trienoic acid moieties of triglycerides.

[b]Calculated from 1-random-2-random-3-random theory.

[c]Results are in mol percent.

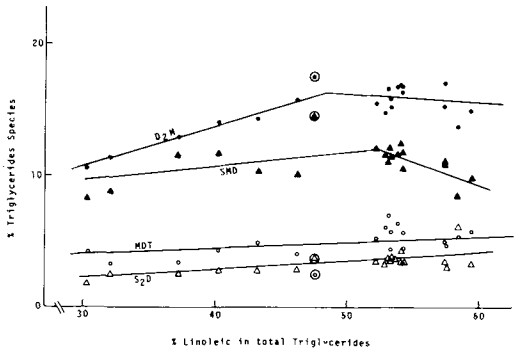

FIG. 9. Percentage of triglyceride species vs. percentage of linoleic acids in soybeans. ○, PI 68-788.

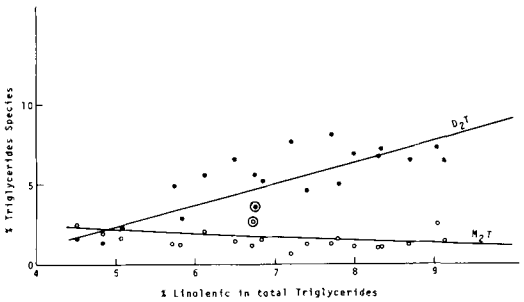

FIG. 10. Percentage of triglyceride species vs. percentage of linoleic acid in soybeans.

circle. PI 68-788 is frequently one of the most deviant points in these plots also, but no other sample deviated from the distribution of points enough to be recognized as a possible genetic deviant.

The relations between the amounts of the triglycerides in the oils and the amounts of their constituent fatty acids generally are positive, as might be expected. For di- and tri-acid triglycerides, one sometimes finds a maximum because, as one fatty acid needed for the triglyceride increases in percentage, another essential fatty acid must decline. Triglycerides occurring at low concentrations sometimes seem to give a zero slope in the graphs. This may be because the slope is so slight that it cannot be detected against experimental error. In Figure 2, M_2S vs. S has an unexpected negative slope. This is because there is a negative correlation between S and M in the samples analyzed. This is not always the case (14). MDT vs. M (Fig. 5) and M_2D vs. D (Fig. 8) also have negative slopes. This is because M and D are negatively correlated. M is often negatively correlated with T, which may account for the negative slope of M_2T vs. T in Figure 10.

Table III also compares the amounts of the triglyceride groups determined by silver ion chromatography with those calculated by the 1-random-2-random-3-random hypothesis using the data reported previously (4). In most instances, the agreement is fairly good. The amounts found for S_3, SMD, S_2T, D_2M, T_2S, T_2M, T_3, M_2D, and MDT usually were less than the calculated values. The amount of D_2T also was less than the calculated value except in instances when D was low. The amounts found for M_2S, M_3, SMT, D_3, and SDT always were

Wells		PI 68-543		PI-70-091		PI 68-788		FA 3043-2		FA 3017-2		FA 3171-2	
Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated	Found	Calculated
–	0.06	–	0.07	–	0.05	–	0.06	–	0.04	–	0.03	–	0.02
1.14	0.98	1.68	1.43	1.49	1.36	2.41	1.77	1.40	1.87	1.59	1.76	1.59	1.77
2.60	1.78	3.74	2.65	3.66	2.89	5.02	4.04	9.24	6.93	10.91	10.36	10.52	9.92
3.87	3.56	4.73	4.22	2.19	3.77	3.99	4.63	2.97	2.43	2.00	1.23	2.45	1.37
3.04	0.81	4.12	1.19	3.55	1.41	3.49	2.15	13.14	6.23	24.55	14.79	23.43	13.43
10.15	10.96	12.22	13.23	12.09	13.49	14.88	15.71	10.99	14.94	8.49	12.42	9.30	12.99
6.94	6.91	6.59	8.39	8.98	9.29	9.04	11.66	15.08	18.89	17.99	25.26	17.84	25.06
15.03	16.09	15.67	15.79	15.59	14.88	14.73	12.86	9.12	7.61	5.05	3.59	5.35	4.08
–	0.36	–	0.39	–	0.36	–	0.47	–	0.26	–	0.16	–	0.16
14.90	19.24	16.65	19.31	15.60	19.96	17.47	19.11	14.19	18.55	10.83	14.10	11.34	15.25
3.21	1.34	1.65	1.51	1.69	1.55	2.81	1.97	3.33	1.99	2.68	1.86	1.94	1.74
1.02	0.97	1.48	1.09	1.06	1.19	2.71	1.64	1.09	2.79	1.98	4.10	2.48	3.61
20.76	17.49	17.21	14.52	16.97	13.97	11.74	9.05	8.99	5.93	6.07	2.58	6.73	3.03
5.16	4.18	5.32	3.81	4.35	3.62	5.88	3.66	2.95	2.15	1.68	1.11	1.82	1.14
4.62	5.61	2.97	5.15	4.19	5.27	2.02	5.67	4.03	5.64	4.18	4.66	3.15	4.47
6.95	7.89	5.09	5.91	5.67	5.62	3.26	4.24	2.90	2.75	1.30	1.29	1.60	1.35
–	0.25	–	0.21	–	0.21	–	0.24	–	0.14	–	0.08	–	0.08
–	0.38	–	0.32	–	0.33	–	0.39	–	0.41	–	0.37	–	0.32
0.62	1.09	0.86	0.76	0.94	0.73	0.57	0.64	0.58	0.41	0.69	0.21	0.46	0.19
–	0.05	–	0.03	–	0.03	–	0.03	–	0.02	–	0.01	–	0.01

higher than the calculated values, and the amounts found for S_2M, S_2D, and D_2S usually were higher than the calculated values. Seemingly, the experimental results were high in combinations of M_2 with M or S, combinations of D_2 with D or S, and combinations of M or D with ST or S_2. Combinations of MD and T_2 with anything (except T_2D) and combinations of S_2 with S and T gave low experimental values. The major discrepancies were observed in the monoacid triglycerides, D_3 and M_3, where the experimental results were significantly greater than the 1-random-2-random-3-random hypothesis predicted.

Some of these deviations from 1-random-2-random-3-random distribution may be accounted for by the results reported by Fehr et al. (15) concerning the change in fatty acid composition of developing soybeans. They found that oleic acid increased during seed development up to 40 days after flowering. After 40 days, oleic acid declined slightly. Linoleic acid increased continually during seed development, reaching its highest values at maturity. Such a developmental sequence would give high values of M_3 and D_3 and a depressed value of glycerides containing both M and D. Calculations based on the data of Fehr et al. (15) for composition and on the data of Simmons and Quackenbush (16) for oil deposition during soybean maturation predict triglyceride compositions that deviate from those calculated from the fatty acid composition of the mature seed by the proper order of magnitude to account for the data in Table III.

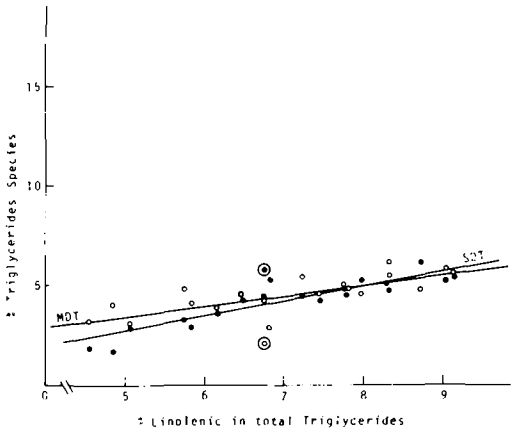

FIG. 11. Percentage of triglyceride species vs. percentage of linolenic acid in soybeans.

Linolenic acid is high during the initial stages of soybean maturation and falls steadily during ripening. Roehm and Privett (11) detected relatively large proportions of T_3, ST_2, and MT_2 during early development, but these disappeared during the later stages. They believed that this was not caused by dilution with other triglycerides formed in greater proportions during the later stages of development; rather, they suggested that the T_3, ST_2, and MT_2 were interesterified into the newly formed triglycerides in some way.

If triglyceride is synthesized roughly according to 1-random-2-random-3-random distribution, the only mutations that would be

very likely would be those that involved the distribution of the amount of fatty acids on the three glycerol positions such as the deviant Pl 68-788 seems to be. But the enzymes assembling triglycerides may control the specific combinations rather than, or in addition to, the proportions of each fatty acid on the three glycerol positions. The discrimination against T_3, T_2M, and T_2S glycerides suggested by Roehm and Privett (11) might be an example of this. If this kind of discrimination exists, mutations involving particular combinations also may be found by examination of additional soybean selections.

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Lipid Composition and Erucic Acid in Rat Liver Cells in Culture

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ABSTRACT

Erucic acid (Δ^{13}-docosenoic acid) was added to fetal calf serum, then fed to rat liver epithelial cells in culture, and uptake measured at intervals over 24 hr. During the first 6 hr. of incubation, uptake of the docosenoic acid was 21 nmoles/hr/mg protein in 7-day cells, and 15 nmoles/hr/mg protein in 14-day cells. Of ^{14}C-labeled erucic acid taken up by the cells in 24 hr, radioactivity measurements showed 60% of the total lipid ^{14}C activity derived from [1-^{14}C] 22:1 in neutral lipid (NL) and 40% in phospholipid (PL); whereas 55% of lipid ^{14}C activity was in NL and 45% in PL when the substrate was [14-^{14}C] 22:1. Within the NL fraction, 75% of ^{14}C activity derived from [1-^{14}C] 22:1 was in triglyceride (TG) and 11% in cholesterol (CHL), while 79% was in TG and 6.5% in CHL when the substrate was [14-^{14}C] 22:1. Triglycerides and cholesteryl esters accumulated in the cells during incubation with erucic acid. Among phospholipids separated by thin layer chromatography, 75% of ^{14}C activity was in lecithin (PC), 10% in phosphatidylethanolamine (PE), 5% in sphingomyelin (SPH), and 1% or less in cardiolipin (DPG). The highest specific activity (SA) was in PC, followed by SPH and PE. Incubation with erucic acid altered fatty acid composition of PC, PE, and SPH, although amounts of phospholipids were unaffected. Gas liquid chromatography analyses detected 18% erucic acid in PC, 2% in PE, and 4-5% in SPH.

INTRODUCTION

The liver of the intact rat, generally, has the capacity to metabolize long chain fatty acids. Its content of erucic acid has been in the range of 2-10% (1-3) following ingestion of a dietary oil rich in this long chain fatty acid. Conclusive evidence for the conversion in rat liver of [14-^{14}C] erucic acid to oleic acid was presented by Carreau (4,5). The hepatic concentration of erucic acid was also lower in the total phospholipid fraction than in the neutral lipid fraction (6). Evidence for the incorporation of erucic acid into rat liver phospholipids was presented by Thoron (7), who showed that erucic acid was preferentially incorporated at position 2 of liver phosphatidylcholine and phosphatidylethanolamine. This was later confirmed by Kramer (3), who found erucic and eicosenoic acids to be present in all lipid classes of liver in rats fed a rapeseed oil diet rich in erucic acid. Similar long term dietary conditions produced a significant elevation of liver triglyceride levels (8).

In studies with beating neonatal rat heart cells in culture (9,10), erucic acid was rapidly taken up and incorporated into major neutral lipid and phospholipid components of the cells. Using radioactivity measurements, two-thirds of the total lipid ^{14}C activity, derived from labeled erucic acid, was present in neutral lipids and one-third in phospholipid (10). the ^{14}C activity in the neutral lipids was principally in triglycerides, while that in the phospholipids was largely in phosphatidylcholine with less in phosphatidylethanolamine and sphingomyelin (10). In animal studies, dietary erucic acid has also been shown to be incorporated into the phospholipids of rat heart mitochondria (11). Experimental findings similar to those obtained in animal feeding trials or with cultured heart cells have not been reported with tissue culture systems involving rat liver cells.

The purpose of the present study was to investigate the effects of unlabeled and ^{14}C-labeled erucic acid on the major neutral lipid and phospholipid components of a cell line of fetal rat liver epithelial cells (12).

MATERIALS AND METHODS

Liver Cell Culture

A line of epithelial-like cells, derived from 18-day-old fetal rat livers (12), was kindly provided by Professor P. Padieu, Laboratoire de Biochimie Medicale, Universite de Dijon, 21033 Dijon, France. The cells were stored in liquid nitrogen at the 20th passage, and were used for experiments after the 22nd or 23rd passage. Stock cultures were grown and maintained in 25 cm^2 plastic culture flasks (Falcon No. 3018) with Ham F10 medium (GIBCO, Grand Island Biological Co., Grand Island, NY), without human serum, but containing 10% fetal calf serum (GIBCO No. 614), penicillin (100 units/ml), streptomycin (100 mcg/ml), and amphotericin B (0.25 mcg/ml). The liver cells were subcultured at weekly intervals according

TABLE I

Distribution of Erucic Acid ^{14}C Activity between Neutral Lipid and phospholipid Components of Rat Liver Cells in Culture

Lipid fraction	^{14}C activity (%)[a]	
	[1-^{14}C] 22:1	[14-^{14}C] 22:1
Neutral lipid (NL)	60.8 ± 0.9[b]	54.9 ± 1.2**[c]
Phospholipid(PL)	39.2 ± 0.9	45.2 ± 1.1**
Ratio:NL/PL[d]	1.6	1.2

[a]Age of cells was 7 days from last passage; incubation time with [^{14}C] 22:1 was 24 hr.
[b]Mean ± standard error of the mean of ten determinations.
[c]**Significantly different from the [1-^{14}C] 22:1 value at $p<0.01$.
[d]Ratio of neutral lipid ^{14}C activity to phospholipid ^{14}C activity.

TABLE II

Distribution of ^{14}C Activity among the Major Neutral Lipid Fractions of Liver Cells after Incubation with [1-^{14}C] or [14-^{14}C] Erucic Acid

Neutral lipid Fraction	Neutral lipid ^{14}C activity[a]			
	[1-^{14}C] 22:1		[14-^{14}C] 22:1	
	DPM/fraction	% Of total DPM	DPM/fraction	% Of total DPM
	(6)	(6)	(5)	(5)
Cholesterol	25966	10.8 ± 0.4[b]	15704	6.5 ± 0.9**[c]
Free fatty acid	19216	8.0 ± 0.5	22494	9.7 ± 0.3
Triglyceride	181201	75.0 ± 0.8	183006	79.0 ± 0.6*[d]
Cholesteryl esters	14769	6.1 ± 0.9	10875	4.7 ± 0.7
Front	307	0.1 ± 0.05	227	0.1 ± 0.01

[a]Age of cells in culture = 7 days from last passage. Incubation time with ^{14}C labeled erucic acid = 24 hr.
[b]Mean ± standard error of the mean. Figures in parentheses denote number of determinations.
[c]Significantly different from the [1-^{14}C] 22:1 value at $p \leq 0.01$.
[d]Significantly different from the [1-^{14}C] 22:1 value at $p \leq 0.05$.

to Chessebeuf et al.(12) with a splitting ratio of 1:9. For experimental use, the cells were grown in plastic Cooper dishes (Falcon No. 3009, diam. 60 mm) containing 4.3 ml of medium (12). Incubation was at 37 C in an atmosphere of 5% CO_2, 95% air, and 100% humidity. The cultures reached confluency within 4-5 days, and were used for experiments after 7 or 14 days incubation. In preparation for experiments, the growth medium was changed at 2-day intervals. Prior to the addition of substrates (5 ml/dish), the medium was removed from 7- or 14-day-old cell cultures and the covers replaced with standard Petri dish lids. Cell protein was measured by the Hartree modification (13) of the method of Lowry et al. (14).

Lipid Analyses

Monolayers of liver cells in culture were harvested in 0.25 M sucrose by gentle scraping of the tissue from the floor of the culture dish with a small rubber policeman. After pelleting the cells by centrifugation (28,000 x g, 4 min), the sucrose was removed and the lipid in the pellet extracted by the method of Bligh and Dyer (15). In some experiments, 20 ml of the sucrose supernatant, which contained some lipid, was solvent-extracted (15), and the chloroform extract concentrated under N_2. Neutral lipid and phospholipid components of the cells and neutral lipids of the sucrose supernatant were separated by thin layer chromatography (TLC) and analyzed according to methods previously described (10). The fatty acids of phosphatidylcholine and phosphatidylethanolamine were methylated and analyzed by gas liquid chromatography (GLC) as described earlier (10). Sphingomyelin fatty acids were prepared for GLC by methylation of the phospholipid with borontrifluoride in flame-sealed glass ampoules (16,17). The identity of GLC peaks was established as previously described (18) by calibration with standard mixtures of methyl esters of fatty acids (Applied Science Laboratories, State College, PA) and from graphic plots of carbon number vs. retention distance.

TABLE III

Phospholipid Phosphorus Values and Phospholipid Radioactivity in Liver Cells Incubated 24 hr with Unlabeled- and ^{14}C-labeled Erucic Acid

Phospholipid[a]	Phospholipid phosphorus (% of phosphorus recovered)			Phospholipid ^{14}C activity			
	Liver cells			[1-^{14}C] 22:1		[14-^{14}C] 22:1	
	Control	22:1[b]	Intact liver	DPM/spot	% Of total DPM	DPM/spot	% Of total DPM
	(8)	(6)	(2)	(5)	(5)	(5)	(5)
DPG	4.4 ± 0.5[c]	4.8 ± 0.6	6.4[d]	1920	0.7 ± 0.1	2018	0.6 ± 0.2
PA	3.1 ± 0.4	3.4 ± 1.3	2.2	4477	1.6 ± 0.2	5338	1.7 ± 0.2
PE	25.6 ± 1.1	22.3 ± 1.5	26.9	24497	8.9 ± 0.4	35334	11.1 ± 0.6*[e]
PS + PI	13.1 ± 0.4	13.1 ± 0.4	13.0	11607	4.2 ± 0.2	19234	6.1 ± 0.3**
PC	40.1 ± 1.8	40.7 ± 1.5	42.7	210027	76.4 ± 1.0	235901	74.4 ± 0.8
SPH	6.8 ± 0.3	7.1 ± 0.3	4.4	16050	5.8 ± 0.5	14589	4.6 ± 0.2
LPC	3.6 ± 0.4	4.0 ± 0.4	2.2	5565	2.0 ± 0.2	3894	1.2 ± 0.2
Origin	2.2 ± 0.4	2.4 ± 0.5	1.2	538	0.2 ± 0.05	656	0.2 ± 0.03
				Total = 274681		Total = 316964	

[a]Abbreviations: DPG, diphosphatidylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine; PS, phosphatidylserine; PI, phosphatidylinositol; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine.

[b]Unlabeled erucic acid = 1 μmole/dish. Age of cells = 7 days from last passage.

[c]Mean ± standard error of the mean. Number of determinations given in parentheses.

[d]Average of two determinations. Livers pooled from each of two Wistar rats, 8-10-days-old.

[e]*Significantly different from the 1-^{14}C value at $p < 0.05$. **Significantly different from the 1-^{14}C value at $p < 0.01$.

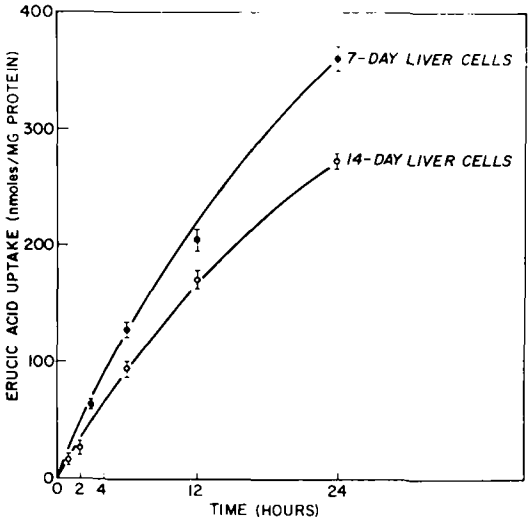

FIG. 1. Relationship between erucic acid uptake and incubation time in hours for 7- and 14-day old cultures of fetal rat liver cells. Each point represents the mean ± SEM of three determinations.

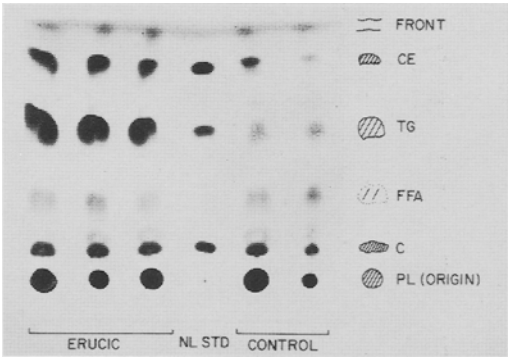

FIG. 2. TLC chromatogram showing relative amounts of neutral lipid classes in sucrose supernatants of liver cell cultures with and without exposure to erucic acid. Each sample represents the lipid extracted (15) from 20 ml of sucrose supernatant from the pooled tissue of 32 culture dishes. Age of cells was 7 days; erucic acid was 1 μmole/dish. Abbreviations: CF, cholesteryl esters; TG, triglycerides; FFA, free fatty acids; C, cholesterol; PL, phospholipids; NL STD=neutral lipid standard mixture of Hojnacki and Smith (29). TLC conditions were those described (10). Visualization: 50% H_2SO_4, 180 C, 10 min.

Radioactive Erucic Acid

Erucic acid was purchased from CEA, Saclay, France, as the carboxyl-[^{14}C]-labeled compound (specific activity, 40 μCi/μmole) or as the [14-^{14}C]-labeled compound (specific activity, 47 μCi/μmole). Labeled fatty acids were prepared for experiments as reported previously (9,10) so that the total erucate per culture dish (5 ml) was 1 μmole. The methods of measurement of radioactivity were those described elsewhere (10). Recovery of ^{14}C, applied to TLC plates, averaged, consistently, 90 to 95%.

RESULTS

A comparison of the rates of uptake of erucic acid by 7- and 14-day-old liver cells in culture is shown in Figure 1. During the first 6 hr, uptake of the docosenoic acid was 21 nmoles/hr/mg protein in 7 day cells, but fell to 15 nmoles/hr/mg protein in 14-day cells.

When incubated with [14-^{14}C] erucic acid, radioactivity measurements showed 54% of [^{14}C] activity to have been taken up from the medium by the liver cells in 24 hr. Of this, 37% was present in the cells and 63% was unaccounted for. The non-lipid fraction contained 16% of the cellular [^{14}C] activity, and the extractable lipid, 84%, of which about 55% was in neutral lipid and 45% in phospholipid.

Total neutral lipid and phospholipid radioactivity values for liver cells, incubated 24 hr with [1-^{14}C] or [14-^{14}C] erucic acid, are compared in Table I. With erucic acid labeled in the 1-position, 61% of the total lipid [^{14}C] activity was in the neutral lipid fraction and 39% in phospholipid, with a neutral lipid to phospholipid ratio of 1:6. When erucic acid was labeled in the 14-position, the neutral lipid [^{14}C] activity value was 55% and the phospholipid value, 45%, with a neutral lipid to phospholipid ratio of 1.2.

TLC was used to separate the major neutral lipids of liver cells after incubation 24 hr with [1-^{14}C] or[14-^{14}C] erucic acid. The distribution of ^{14}C activity among various neutral lipid fractions is shown in Table II. Of the ^{14}C activity derived from [1-^{14}C] erucic acid, 75% was in triglycerides and 11% in cholesterol. By comparison, the ^{14}C activity in the triglyceride fraction was significantly higher (79%) and in the cholesterol fraction, significantly lower (6.5%), when the substrate was erucic acid labeled at the double bond.

During the harvesting of liver cells from cultures incubated with erucic acid, a fatty layer regularly appeared in the sucrose supernatant after centrifugation, and correlated with the appearance of numerous, dark, cytoplasmic granules in cells exposed to erucic acid. An analysis of the sucrose-lipid layer by TLC is shown in Figure 2. The amounts of tissue, from which extracts were derived, were the same in control and erucic-treated cultures. A large increase in triglycerides and in the cholesteryl ester fraction was seen in the sucrose-lipid extracts prepared from cells exposed to erucic acid.

TABLE IV

Major Fatty Acids of Phosphatidylcholine, Phosphatidylethanolamine, and Sphingomyelin in Rat Liver Epithelial Cells Before and After Incubation with Erucic Acid.

	Major fatty acids, wt % values[a]					
	Phosphatidylcholine		Phosphatidylethanolamine		Sphingomyelin	
Fatty acid	Control	Erucic[b]	Control	Erucic	Control	Erucic
16:0[c]	36.0±0.8[d]	28.0±1.2*[e]	10.6±1.0	9.4±0.7	44.0±1.4	44.6±2.7
16:1	6.9±0.4	5.8±0.5	2.5±0.2	2.2±0.3	1.9±0.1	3.3±1.1
18:0	12.4±0.4	7.4±0.2**	32.8±2.1	31.8±2.6	26.0±2.8	24.2±2.2
18:1	24.8±1.0	26.4±1.8	16.8±1.0	17.7±2.1	7.2±2.4	6.4±1.0
18:2	1.7±0.2	1.8±0.1	1.0±0.1	1.3±0.2	2.1±0.6	1.8±0.2
20:1	2.5±1.0	2.1±0.9	1.1±0.3	1.1±0.1	6.0±0.5	4.3±0.5
20:3	3.4±1.4	4.1±3.6	4.7±1.4	7.4±2.7	—	—
20:4	5.6±1.7	3.0±0.5	15.5±0.8	12.6±1.9	—	—
22:1	—	18.3±1.5	—	2.2±0.7	—	4.6±0.9
22:2	—	—	—	—	3.6±1.8	3.4±2.2
22:6	1.1±0.4	1.4±0.3	3.7±1.1	3.1±1.0	—	—
24:0	—	—	—	—	4.2±1.1	4.9±1.8
24:1	—	—	—	—	3.5±2.2	2.9±0.8

[a]Contained minor to trace amounts of 14:0, 15:0, 17:0, 17:1, 20:0, 20:2, 22:3, and 22:5.
[b]Erucic acid concentration was 1 μmole/culture dish. Incubation time was 24 hr.
[c]Number of carbon atoms:number of double bonds.
[d]Mean ± standard error of the mean of four determinations.
[e]**Significantly different from the control value at $p<0.01$. *Significantly different from the control value at $p<0.05$.

To determine the distribution of erucic acid or its metabolites among individual phospholipids, liver cell cultures were incubated 24 hr with unlabeled erucic acid or with the same fatty acid labeled at the carboxyl position or at the double bond, and then were extracted of lipid. The distribution of lipid phosphorus and of radioactivity in each phospholipid fraction was measured after separation by TLC. The proportions of phospholipids in liver cells were unaffected by erucic acid and resembled closely those of intact rat liver (Table III). Phosphatidylcholine (PC) was the most abundant phospholipid (41% of total lipid phosphorus) and contained 75% of the radioactivity derived from [1-^{14}C] or [14-^{14}C] erucic acid. Phosphatidylethanolamine (PE) was the second most abundant phospholipid (25% of phosphorus) but contained only 9-11% of the ^{14}C activity. Other ^{14}C activity values were sphingomyelin (SPH), 5-6%; lysophosphatidylcholine (LPC), 1-2%; and cardiolipin (DPG), <1%; With the exception of a small increase in ^{14}C activity of the PE and PS+PI fractions, incubation with [14-^{14}C] 22:1 gave similar values to those obtained with the [1-^{14}C] substrate.

The incorporation of erucic acid or its metabolites into the major phospholipids of rat liver cells in culture was compared by measuring the specific activities of SPH, PC, and PE after incubation with [1-^{14}C] of [14-^{14}C] erucic acid. The highest specific activity was in PC followed by SPH and PE, regardless of the [^{14}C] erucic acid substrate to which the cells were exposed.

The relationship of erucic acid to liver cell phospholipids was further investigated by GLC analyses of the fatty acid composition of PC, PE, and SPH before and after incubation with erucic acid (Table IV). After 24 hr, erucic acid was 18% of the fatty acids of PC, about 5% of the fatty acids of SPH, and less than 3% of the fatty acids of PE. The large incorporation of erucic acid into PC was accompanied by changes in the fatty acid pattern shown by a significant decrease in percentage of palmitic (16:0) and stearic (18:0) acids. Statistically significant differences in fatty acid concentrations were not seen in PE or in SPH following incubation with erucic acid.

DISCUSSION

In rat liver cells in culture, the amount of erucic acid taken up from the medium in 24 hr was comparable to that reported for rat myocardial cells (10), although with liver cells the initial rate of uptake was lower. The decline in erucic acid uptake by 14-day liver cells could be attributed to gradual dedifferentiation of the cells in culture (19).

That cultured liver cells differed from cultured heart cells (10) in the manner in which they metabolized long chain fatty acids was suggested: (a) by the distribution pattern of erucic acid ^{14}C activity among intracellular components; (b) by the greater incorporation of ^{14}C activity into phospholipids and the less incorporation into neutral lipids; and (c) by the

larger amount of ^{14}C activity unaccounted for after incubation 24 hr with this ^{14}C-labeled docosenoic acid. The rapid loss of $^{14}CO_2$ from tissue culture preparations of rat heart and liver cells during incubation with [^{14}C] erucic acid has been well documented (9). Approximately 66% of phospholipid ^{14}C activity, derived from [^{14}C] erucic acid, was in PC of heart cells (10), compared to 75% in liver cells. A higher uptake of erucic acid into liver cell phospholipids was further indicated by GLC which showed that, for PC, erucic acid was 18% of the fatty acids in liver cells, but less than 9% in heart cells. In comparison to heart cells (10), a smaller amount of ^{14}C activity was located in the triglyceride fraction of liver cells, and this appeared to reflect a more rapid turnover of this long-chain fatty acid in liver. This observation may also be related to the early accumulation of triglycerides in cardiac tissue, but not in liver, of rats fed a semisynthetic diet rich in erucic acid (20,21). In contrast, long term feeding studies with erucic acid have shown an increase over 5 months in triglyceride levels in rat liver but not in heart (8). The present findings suggest that erucic acid stimulates the production of triglycerides and promotes the accumulation of cholesteryl esters in tissue culture preparations of fetal rat liver cells.

When incubated with [1-^{14}C] 22:1, liver cell cultures showed a two fold increase in ^{14}C activity of the cholesterol fraction compared to that of cells incubated with [14-^{14}C] 22:1. indicating that the cholesterol fraction may in part be derived from short chain metabolites of erucic acid. Evidence in support of this possibility was presented by Carroll (22) who showed that erucic acid promoted incorporation of [1-^{14}C] acetate into cholesterol by rat liver slices. Among liver cell phospholipids, large differences in ^{14}C activity could not be attributed to a difference in position of the ^{14}C label within the erucic acid molecule.

Amounts of individual liver cell phospholipids, while unaffected by erucic acid, resembled those of intact liver from 10-day-old rats. With the exception of a lower value for phosphatidylcholine, the present results were in general agreement with data reported for intact liver by Parker and Peterson (23), Bergelson et al.(24), and by Wuthier (25).

In the absence of erucic acid, the fatty acid composition of PC and PE from rat liver cells was similar to intact rat liver values (18,26) for palmitic and stearic acid, but differed in having lower values for polyenoic fatty acids. With the exception of twofold higher values for palmitic and stearic acid, the amounts of fatty acids in liver cell SPH were generally lower than those of intact rat liver (26).

The large incorporation of erucic acid displayed by phosphatidylcholine, the most abundant phospholipid in liver cells in culture, is of considerable interest since this phospholipid is a major structural component of cell membranes (27). The behavior towards erucic acid shown by the phospholipids of liver cells in culture was similar to that previously observed (10) with newborn rat heart cells in which the myocardial PC fraction also displayed the largest incorporation of erucic acid, followed by that of PE and SPH. Animal feeding studies have also shown that dietary erucic acid can be incorporated into rat heart phospholipids (11,28). The relative incorporation of erucic acid into PC and PE of rat liver cells in culture was reflected in the phospholipid specific activity values and was in agreement with animal studies by Thoron (7), who found, after 7 months, that erucic acid was 10% of the fatty acids of PC and 6% of the fatty acids of PE in livers of rats fed a rapeseed oil diet rich in erucic acid. Other studies (3) have shown incorporation of small amounts of this docosenoic acid into rat liver PC and PE fractions. In the work reported here, however, the incorporation of significant amounts of erucic acid into major phospholipids of liver cells would suggest that this docosenoic acid may have a direct effect on membrane associated metabolic functions.

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Alveolar Lavage and Lavaged Lung Tissue Phosphatidylcholine Composition During Fetal Rabbit Development

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ABSTRACT

Pulmonary surfactant, the major surface-active component of which in the adult is dipalmitoylglycerophosphocholine, can be obtained by lavaging lungs with physiological saline. We have previously shown that there is an increase in the amount of phosphatidylcholine in fetal rabbit lung lavage during the latter part of gestation. We have now measured the amount of disaturated phosphatidylcholine as well as the fatty acid composition of phosphatidylcholine in lung lavage from fetal rabbits during the period 27 days' gestation to full term (31 days). There was no developmental change in the amount of disaturated phosphatidylcholine during the period examined. About 50% of the total phosphatidylcholine was disaturated which is approximately the same as in adult rabbit lung lavage. The fatty acid composition, however, did change. There was an increase in the amount of 16:0 from about 20% of the total fatty acids in phosphatidylcholine at 27-28 days to about 60% at full term, after birth, and in the adults. There was a corresponding decrease in the amounts of 14:0, 18:0, and longer chain fatty acids, most of which were saturated. In the lavaged lung tissue, there was a 2.6-fold increase in the percentage of phosphatidylcholine which was disaturated during the period 27-31 days' gestation. It had not decrease to the adult value 24 hr after birth.

INTRODUCTION

The lung is lined with surface-active material, pulmonary surfactant, which is believed to lower surface tension at the air-liquid interface and prevent alveolar collapse (1). Surfactant can be obtained from the lung by lavaging with saline. It has been shown to be largely phospholipid of which phosphatidylcholine is the most abundant component (1,2). Dipalmitoylglycerophosphocholine is believed to be the major surface-active component of pulmonary surfactant (1,2).

Insufficient surfactant at birth can lead to the respiratory distress syndrome of the newborn, the major cause of morbidity and mortality among premature infants in developed countries (3,4). The fetal lung begins to produce appreciable amounts of surfactant towards the end of gestation (2,3), and it has been shown that corticosteroids (3) and thyroxine (5) can accelerate fetal lung maturation and surfactant production.

We have previously measured the phospholipid content and composition of lung lavage during the latter part of gestation in fetal rabbits and shown that there is a fourfold increase in the amount of total phospholipid between 27 and 31 (full term) days' gestation (6). During the same period, phosphatidylcholine increased from 29% of the total phospholipid to 80% (6). We have also shown that cortisol (7) and thyroxine (8) increase the amount of phosphatidylcholine in fetal rabbit lung lavage. However, there is little information on the nature of the molecular species of phosphatidylcholine in lung lavage during fetal development although detailed studies have been carried out on intact lung tissue (9).

The purpose of the present study was to examine changes in the amount of saturated phosphatidylcholine and in the fatty acid composition of phosphatidylcholine from lung lavage and lavaged lung tissue during development of the fetal rabbit. Such basic information is essential in elucidating the mechanism of surfactant production and the effects of hormones thereon during fetal development.

MATERIALS AND METHODS

Timed pregnant rabbits (New Zealand White) were purchased from Glochester Rabbitry, Chepachet, RI. The doe was sacrificed with intravenous sodium pentobarbital (80 mg/kg). The fetuses were immediately delivered by cesarean section and sacrificed with intraperitoneal sodium pentobarbital (30 mg) prior to breathing, while still in their amniotic sacs. Newborn animals were similarly sacrificed 1-24 hr after delivery by cesarean section, during which time they were kept in an incubator at constant temperature (31 C) and humidity. The lungs were lavaged in situ, via a tracheal cannula, with five 1-3 ml aliquots of 0.9%

TABLE I

Disaturated Phosphatidylcholine Content of Lung Lavage and Lavaged Lung Tissue from Fetal, Newborn, and Adult Rabbits[a]

		Lung lavage	Lung tissue
		Disaturated phosphatidylcholine as % of total phosphatidylcholine	
Fetuses	Gestational age (days)		
	27	49.3 ± 1.9	27.0 ± 3.2
	28	55.5 ± 0.9	34.0 ± 4.5
	29	46.0 ± 5.3	34.1 ± 3.8
	30	50.2 ± 4.4	35.2 ± 3.3
	31	52.8 ± 3.0	41.6 ± 2.6
Newborns	Period after birth (hours)		
	1		42.2 ± 1.8
	3		47.3 ± 1.5
	6		57.6 ± 0.8
	18		45.8 ± 3.9
	24		45.2 ± 1.7
Adult[b]		54.0 ± 1.4	28.6 ± 1.3

[a]The data are the means (±SE) from 3-6 experiments in each of which lavage and tissue from all fetuses in 1-2 litters were combined. The newborn and adult data are from 3-5 animals.

[b]The adult data are from a previous study (14).

NaCl. The lavage liquid was centifuged at 1000 x g to remove cellular material. Lipids were extracted from the lyophilized lavage and tissue as described previously (6,10).

Phosphatidylcholine was isolated by thin layer chromatography (TLC) on Quantum LQD plates (Quantum Industries, Fairfield, NJ) in chloroform-methanol-7 M NH_4OH (60:35:4, by vol). It was eluted from the gel and quantitated by phosphorus assay as described previously (10).

Phosphatidylcholine was saponified with methanolic KOH (11). The fatty acids were methylated, and the methyl esters were analyzed by gas liquid chromatography as described previously (12).

Disaturated phosphatidylcholine was separated from total phosphatidylcholine from lung tissue following osmium tetroxide treatment by the method of Mason et al. (13) as described previously (14). Lavage disaturated phosphatidylcholine was similarly measured except that it was separated from the oxidized phosphatidylcholine by TLC on Quantum LQD plates in chloroform-methanol-7 M NH_4OH (70:30:3.5, by vol) (13). Quantitation was by the ultramicro phosphorus assay described by Bartlett (15). In this procedure, 86% of standard dipalmitoylglycerophosphocholine and 1.5% of standard dioleoylglycerophosphocholine were recovered in the phosphatidylcholine band.

Standard phospholipids were purchased from Serdary, London, Ontario, Canada and standard fatty acid methyl esters from Supelco, Bellefonte, PA. Other chemicals were reagent grade or better.

RESULTS

In the lung lavage from fetal rabbits, about half of the total phosphatidylcholine was disaturated throughout the developmental period examined (Table I). A similar amount of phosphatidylcholine is disaturated in lung lavage from the adult rabbit.

The fatty acid composition of phosphatidylcholine from fetal and newborn rabbit lung lavage is shown in Table II. In agreement with the finding with respect to disaturated phosphatidylcholine, there was little change in the total amount of saturated fatty acids during development. The composition of the fatty acids, however, did change. There was an increase in the amount of 16:0 and a corresponding decrease in the amounts of 14:0, 18:0, and longer chain (> 18 carbon atoms) fatty acids from 27 days' gestation to full term. The fatty acid composition of lung lavage phosphatidylcholine at 31 days' gestation and 6 hr after birth was similar to that of adults (12). At 31 days' gestation and after birth, as well as in adults (12), 16:0 accounted for about 60% of the total and 90% of the saturated fatty acids. Thus, the bulk of disaturated phosphatidylcholine in lung lavage at this developmental stage must be dipalmi-

TABLE II

Fatty Acid Composition of Phosphatidylcholine from Lung Lavage of Fetal and Newborn Rabbits[a]

Fatty acid methyl esters	Gestational age (days)					6 Hr after birth at 31 days' gestation
	27	28	29	30	31	
n	2	3	2	3	4	2
14:0	14.2 ± 2.0	10.5 ± 4.8	3.6 ± 2.5	1.2 ± 0.5	3.4 ± 0.3	5.6 ± 0.1
14:2	1.6 ± 1.1	0.2 ± 0.2				
15:0	0.6 ± 0.2	8.6 ± 4.1	0.2 ± 0.2	0.1 ± 0.1	0.5 ± 0.1	0.3 ± 0.3
16:0	23.0 ± 3.0	19.9 ± 5.1	52.1 ± 1.0	49.1 ± 4.9	58.2 ± 1.4	59.4 ± 1.6
16:1	3.9 ± 1.3	12.1 ± 2.9	12.1 ± 1.0	14.0 ± 1.8	15.9 ± 0.3	15.6 ± 1.5
18:0	7.9 ± 1.5	12.8 ± 1.6	11.0 ± 2.3	11.0 ± 2.4	0.9 ± 0.2	1.1 ± 0.1
18:1	6.9 ± 1.3	8.9 ± 1.2	10.0 ± 0.0	10.6 ± 1.9	5.4 ± 0.3	7.1 ± 0.2
18:2	7.8 ± 1.4	10.1 ± 4.1	6.0 ± 2.6	9.1 ± 2.2	12.2 ± 1.7	8.5 ± 0.4
18:3	2.3 ± 0.6	1.9 ± 1.0	1.4 ± 1.4	1.5 ± 1.2	0.8 ± 0.2	0.6 ± 0.1
19:0	2.9 ± 0.9	1.7 ± 0.9				
20:0	7.2 ± 1.0	2.1 ± 1.5			1.9 ± 0.5	0.8 ± 0.5
21:0	8.1 ± 0.8	2.9 ± 2.1	1.9 ± 0.7	2.8 ± 2.0		
22:0	3.2 ± 1.0	1.5 ± 0.8				
20:4	5.3 ± 0.6	0.5 ± 0.5	1.0 ± 1.0	0.6 ± 0.5		
23:0	4.0 ± 1.3	2.8 ± 1.7				
Unidentified	1.7 ± 0.1	3.5 ± 0.7	0.8 ± 0.8		1.1 ± 0.2	0.9 ± 0.2
Total saturated	71	63	69	64	65	67
16:0 as % of total saturated	32	32	76	77	90	88

[a]The data are expressed as a percentage by wt (± SE) of the total fatty acid methyl esters. Fatty acid methyl esters were tentatively identified on the basis of relative retention times in comparison to those of standards. n = Number of litters. The values from each litter were the means of 2-3 determinations.

TABLE III

Fatty Acid Composition of Phosphatidylcholine from Lavaged Lung Tissue of Fetal Rabbits[a]

Fatty acid methyl ester	Gestational age (days)					
	24[b]	27	28	29	30	31
n	4	3	3	3	3	3
14:0	3.3 ± 0.7	0.8 ± 0.4	1.6 ± 0.1	2.5 ± 0.5	2.3 ± 0.1	3.2 ± 0.3
14:2	1.7 ± 0.6	trace	1.7 ± 0.6	0.9 ± 0.8	0.6 ± 0.2	trace
15:0	trace	trace	trace	0.6 ± 0.1	0.5 ± 0.2	0.8 ± 0.1
16:0	35.9 ± 5.0	39.2 ± 1.5	47.0 ± 0.7	49.0 ± 1.7	49.1 ± 1.7	51.5 ± 0.6
16:1	7.2 ± 0.7	4.4 ± 0.8	5.2 ± 0.1	6.5 ± 0.7	7.2 ± 0.6	8.4 ± 0.4
18:0	6.8 ± 0.7	7.5 ± 0.7	5.8 ± 0.4	5.6 ± 0.6	4.7 ± 0.1	4.7 ± 0.0
18:1	24.9 ± 1.2	23.8 ± 2.2	17.5 ± 1.0	15.6 ± 0.6	15.0 ± 0.7	13.1 ± 0.6
18:2	13.9 ± 2.0	14.6 ± 1.2	11.5 ± 0.4	11.3 ± 1.1	11.6 ± 0.3	12.2 ± 0.2
18:3	trace	1.2 ± 0.1	0.9 ± 0.2	0.5 ± 0.3	0.9 ± 0.2	0.8 ± 0.1
22:0	trace	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.1
20:4	3.4 ± 1.0	6.1 ± 1.3	6.8 ± 0.5	5.7 ± 0.7	6.5 ± 1.4	3.6 ± 1.4
Others	2.9 ± 1.4	1.7 ± 0.1	1.2 ± 0.2	1.0 ± 0.2	0.8 ± 0.2	1.1 ± 0.1

[a]Trace, < 0.5%. Other details as in Table II.

[b]The lungs from the 24 day fetal rabbits were not lavaged prior to lipid extraction.

toylglycerophosphocholine. (The fatty acid composition of the disaturated species was not determined because of the small amounts of material available for analysis). At 29 and 30 days' gestation, 16:0 accounted for about 50% of the total and 76-77% of the saturated fatty acids so that, even at this stage of development, dipalmitoylglycerophosphocholine would be the major disturated species. At 27 and 28 days, however, 16:0 accounted for only about 20% of the total and 30% of the saturated fatty acids. Thus, at this developmental stage, dipalmitoylglycerophosphocholine is probably a minor component of lung lavage phosphatidylcholine. Long chain fatty acids, largely saturated, accounted for 31% of the total at 27 days' gestation and 12% at 28 days but only 2-3% at 29-31 days. Arachidonic acid (20:4), which is characteristically absent from surfactant, accounted for 5% of the total at 27 days. 0.5-1% at 28-30 days, and was not seen at 31 days or after birth. There was an increase in the amount of 16:1 from 4% of the total at 27 days to 12% at 28-29 days and to about 16% at full term.

In the lavaged lung tissue, disaturated phosphatidylcholine increase from 27% of the total phosphatidylcholine at 27 days' gestation to 42% at 31 days (Table I). It increased even further after birth and did not decrease to the adult value during the period examined. The fatty acid composition of phosphatidylcholine from lavaged lung tissue is shown in Table III. There was little change during the period 24-31 days' gestation other than an increase in the amount of 16:0 from 36% of the total fatty acids to 52% and a corresponding decrease in the amount of 18:1 from 25% to 13%. These changes in fatty acid composition are consistent with the developmental increase in disaturated phosphatidylcholine. By 31 days' gestation, the fatty acid composition of lung tissue phosphatidylcholine was similar to that of adults (14).

DISCUSSION

Surfactant appears to be synthesized by the fetal lung towards the end of gestation (2) and largely stored, presumably in lamellar inclusion bodies in type II alveolar epithelial cells (16), until after birth when it is released in large quantity into the alveoli (6,17). It has been shown that there is an increase in the amount of phospholipid, particularly phosphatidylcholine, in lung lavage during the latter part of gestation in the fetal rabbit (6) and lamb (18). However, the total amount in the lavage even at full term accounts for no more than 1% of the total pulmonary phospholipid or phosphatidylcholine (8). One day after birth, on the other hand, 7% of total pulmonary phospholipid and 10% of total pulmonary phosphatidylcholine is recovered in the lavage (8).

There are also changes in the composition of lung lavage phospholipid during development. At 27 days' gestation in the rabbit, for instance, phosphatidylcholine accounts for only 29% of the total phospholipid while sphingomyelin accounts for 38% (6). After birth (6) and in the adult (10), phosphatidylcholine accounts for at least 80% of the total but sphingomyelin accounts for less than 3%. Similarly, as shown in this paper, there are changes in the fatty acid composition of phosphatidylcholine from lung lavage during fetal development. From these studies, it is apparent that dipalmitoylglycerophosphocholine, the major surface-active component of pulmonary surfactant in adults (1,2),

is only a relatively minor component of lung lavage phospholipid before about 29 days' gestation in the rabbit. It is of interest that the surface activity of lung lavage phosphatidylcholine also begins to increase at the same gestational age (19).

There are few previous studies on the fatty acid composition of lung lavage phosphatidylcholine during fetal development. Fijiwara et al. (18) reported that the amount of 16:0 in phosphatidylcholine from fetal lamb lung lavage increased with increasing gestational age. From fatty acid analysis, these workers calculated that the percentage of minimum disaturated phosphatidylcholine increased during the same period (18). Gluck et al. (20) also reported that there was more 16:0 in phosphatidylcholine from lung lavage of fetal rabbits at 31 days' gestation than in that from those at 23 days. We have carried out studies on tracheal and gastric liquids from newborn humans at birth (sources of alveolar liquids) and found that phosphatidylcholine in the materials from premature infants contained less 16:0 and more 18 carbon fatty acids than in that from full term infants (21). Similar data has been reported for amniotic fluid (another source of fetal surfactant) from humans (22-25) and sheep (26).

An increase in the amount of pulmonary disaturated phosphatidylcholine during fetal development has also been reported by others. Fujiwara et al. (18) and Brumley et al. (27) reported a developmental increase in the amount of disaturated phosphatidylcholine in lavaged and intact fetal lamb lung, respectively. Soodsma et al. (9) recently reported increases in the amounts of disaturated phosphatidylcholine and dipalmitoylglycerophosphocholine in intact lungs from fetal rabbits during the period 23.5 to 30.5 days' gestation.

The increased amount of disaturated phosphatidylcholine in lavaged lung tissue with increasing gestational age may reflect increased synthesis and storage of surfactant prior to birth. It is of interest that the amount of disaturated phosphatidylcholine in the lavaged tissue does not decrease immediately after birth when there is a tremendous increase in the amount of phosphatidylcholine – about half of which is disaturated – in the lavage (6,17). It is possible that not all of the stored surfactant is released into the alveoli in the first 24 hr after birth. The lower level of disaturated phosphatidylcholine found in the adult lung tissue may not be reached for some time after birth.

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Analysis of Autoxidized Fats by Gas Chromatography-Mass Spectrometry: III. Methyl Linolenate[1]

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ABSTRACT

The gas chromatography-mass spectrometry (GC-MS) method developed in the preceding papers was extended to the analysis of autoxidation products of methyl linolenate. Four isomeric hydroxy allylic trienes with a conjugated diene system were identified after reduction of the linolenate hydroperoxides. All eight geometric *trans,cis-* and *trans, trans-*conjugated diene isomers of these hydroxy allylic compounds were identified and partially separated by GC of the trimethylsilyl (TMS) ether derivatives. The proportion found of 9- and 16-hydroperoxides was significantly higher (75-81%) than the 12- and 13-hydroperoxides (18-25%). The tendency of the 12- and 13-hydroperoxides to form cyclic peroxides, cyclic peroxide-hydroperoxides, and prostaglandin-like endoperoxides was supported by indirect evidence for the presence of 9,10,12- and 13,15,16-trihydroxyoctadecanoate in hydrogenated derivatives of the highly oxygenated products. The quantitative GC-MS method was used to determine the relative contribution of linolenate, linoleate, and oleate in mixtures to the formation of hydroperoxides.

INTRODUCTION

In the first two papers in this series (1,2), structural investigations were made of autoxidation products of methyl oleate and linoleate by gas chromatography-mass spectrometry (GC-MS). We showed that this analytical tool is powerful for qualitative studies, but quantitative studies require standardization with authentic synthetic compounds. Quantitative GC-MS was also used as a method for determining isomeric hydroperoxide composition of autoxidized mixtures of methyl oleate and linoleate (2). This paper reports an extension of these studies to the autoxidation products of methyl linolenate and to the analysis of its autoxidized mixtures with methyl linoleate and oleate.

EXPERIMENTAL PROCEDURES

Methyl linolenate was prepared from methyl esters of linseed oil and purified by counter-double current distribution (3). It analyzed 99.9% linolenate by GC.

Procedures for autoxidation, analyses, KI and $NaBH_4$ reduction, catalytic hydrogenation, silylation, and GC-MS were the same as before (1). Catalytic hydrogenation of autoxidized linolenate with PtO_2 in 95% EtOH caused no apparent hydrogenolysis of oxidation products because GC analysis showed no increase in stearate. Authentic 9-, 13-, and 16-hydroxyoctadecanoate were derived by $NaBH_4$ reduction of the corresponding keto esters synthesized by literature methods (4). Methyl 12-hydroxyoctadecanoate was derived from ricinoleate by catalytic hydrogenation and purified chromatographically (5). Methyl 9,10,12-trihydroxyoctadecanoate was prepared by hydroxylation of either ricinoleic acid with alkaline $KMnO_4$ to obtain the erythro acids, or methyl ricinoleate with formic acid-H_2O_2 to obtain a mixture of diastereoisomers (6). For these polyhydroxy compounds to achieve com-

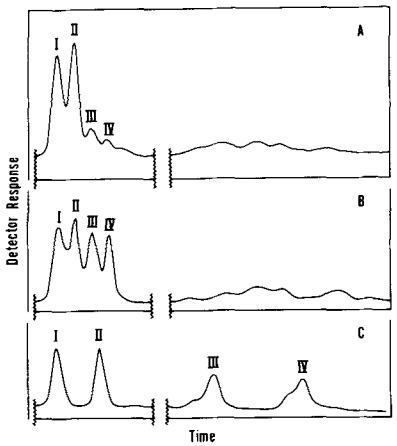


FIG. 1. Gas chromatogram of silyl ethers from hydroxy derivatives of methyl linolenate autoxidized at room temperature to a peroxide value of 1315. A. $NaBH_4$ reduced. B. KI reduced. C. Catalytically hydrogenated.

[1]Presented at the AOCS Meeting, New York, May 1977.

TABLE I

Mass Spectral Data

Peak	Characteristic fragments m/e (relative abundance)	Identification[a,b] (C-18 OTMS methyl esters)
Figure 1A	$NaBH_4$ reduced-oxidized linolenate	
I	183 (79.7), 365 (2.0) M-15, 380 (1.3) M	12-OH-triene (B)[c]
	223 (23.4)	9-OH-triene (A)
	311 (56.3)	13-OH-triene (C)
II	183 (78.7),[d] 365 (2.0) M-15, 380 (1.7) M	16-OH-triene (D)
	351 (15.9)	
Figure 1B	KI reduced-oxidized linolenate	
I	183 (67.6), 365 (1.8) M-15, 380 (1.5) M	12-OH-triene (B)[c]
	223 (30.1)	9-OH-triene (A)
	311 (55.8)	13-OH-triene (C)
II	183 (95.4), 365 (1.5) M-15, 380 (1.5) M	12-OH-triene (B)[e]
	351 (13.8)	16-OH-triene (D)
III	223 (45.3), 365 (1.7) M-15, 380 (2.5) M	9-OH-triene (A)
	311 (56.7)	13-OH-triene (C)
IV	183 (75.3),[c] 365 (1.6) M-15, 380 (1.5) M	16-OH-triene (D)
	351 (18.4)	
Figure 1C	Hydrogenated-oxidized linolenate	
I	229 (93.9), 259 (100), 371 (4.3) M-15	9-OH-ane
	187 (26.5), 301 (22.7)	12-OH-ane
	173 (12.6), 315 (8.3)	13-OH-ane
II	131 (100), 357 (27.4), 371 (1.7) M-15	16-OH-ane
III	187 (100), 259 (37.2), 503 (1.0) M-59	9,10,12-triOH-ane
IV	131 (36.4), 315 (100), 503 (1.0) M-59	13,15,16-triOH-ane

[a]Based on comparison with reference compounds: methyl 9-, 12-, 13-, 16-hydroxyoctadecanoate, methyl 9,10,12-trihydroxyoctadecanoate, and by analogy with reported fragmentation schemes (9,10).

[b]See structures and fragmentation given in text.

[c]Due to *cis,trans*-12-OH-triene.

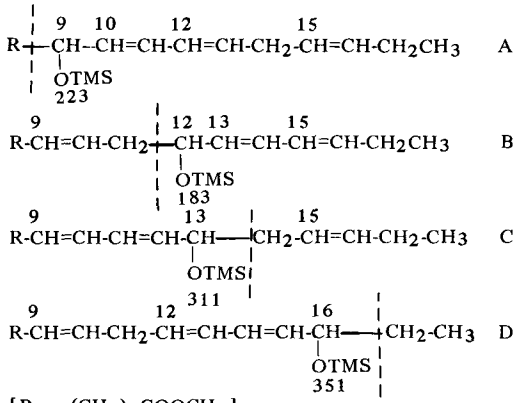
[d]Rearrangement fragment from D.

[e]Due to both *trans,trans*-12-OH-triene and to rearrangement from D.

plete silylation, it was necessary to use pyridine, hexamethyl disilazane, and trimethylchlorosilane (7).

RESULTS

Four isomeric allylic hydroxy trienes with a conjugated *trans,cis/cis,trans* diene system are formed by reducing the corresponding linolenate hydroperoxides (8). The structure of the trimethylsilyl ether (OTMS) derivatives and the expected MS fragmentation are as follows:



When methyl linolenate was autoxidized at room temperature to minimize the formation of *trans,trans*-diene isomers, two prominent peaks I and II were obtained on GC after $NaBH_4$ reduction and silylation (Fig. 1A). MS showed that peak I is due to a mixture of 9-, 12-, and 13-OTMS esters (A, B, and C) by the respective masses 223, 183, and 311; and that peak II is due to the 16-OTMS ester (D) (Table 1). The expected fragment mass for this isomer D, m/e 351, is accompanied by m/e 183, due to the 12-OTMS isomer B. Apparently, on mass spectrometry, there is a rearrangement between D and B, and the fragment of m/e 183 is favored because it has only one oxygenated function or because it tends not to form the high energy ethyl radical. Any corresponding rearrangement expected between the 9- and 13-OTMS isomers (A⇔C) could not be detected because these two isomers are not separated and emerge together within peak I. Identifications of the four hydroxy isomers is confirmed below by analyses of the saturated derivatives.

When methyl linolenate was autoxidized at 60 C, peaks III and IV increased in relative size. These peaks became especially prominent when

KI was used instead of $NaBH_4$ as the reducing agent (Fig. 1B). In our previous paper (2), we observed that the 9- and 13-dienols from autoxidized linoleate are separated by GC according to the conjugated diene configuration, the *trans, trans*-isomers having a longer retention than the *cis,trans*-isomers. Also, it was shown that KI reduction results in an increase in the relative proportion of the *trans,trans*-dienol isomers. Infrared analyses showed that the ratio of the bands at 983 *(trans,trans* plus *cis,trans)* and 945 cm^{-1} *(cis,trans)* was greater in the KI-reduced sample (12.0) compared to the $NaBH_4$-reduced sample (6.5). On the basis of these observations and of the MS data (Table I), the following structural assignments can be made for the gas chromatogram in Figure 1B. Peak I: mixture of *trans,cis*-9-OTMS (m/e 223), -12-OTMS (m/e 183) and -13-OTMS (m/e 311) esters; peak II: mixture of *trans,trans*-12-OTMS (m/e 183) and *trans,cis*-16-OTMS (m/e 351 and m/e 183 by rearrangement); peak III: mixture of *trans, trans*-9-OTMS (m/e 223) and -13-OTMS (m/e 311); and peak IV: *trans,trans*-16-OTMS (m/e 351 and m/e 183 by rearrangement).

The more polar peaks of longer retention than peak IV (Fig. 1A and 1B) are more difficult to identify without proper reference compounds. However, significant mass fragment of m/e 131 and 259 suggests the presence of polyhydroxy esters with OTMS substituents on C-16 and C-9, respectively. Less abundant fragments at m/e 183 and 311 also indicate the presence of polyhydroxy esters with OTMS substitutents on C-12 and C-13, respectively.

After double bond hydrogenation of the autoxidized linolenate, the gas chromatogram showed four well-separated peaks (Fig. 1C). MS identifications (Table I) were confirmed by comparison of chromatographic (GC and TLC) and MS data with those of authentic compounds and by mass chromatography (1,2). The 9-, 12-, and 13-OTMS saturated esters are partially separated in peak I, and the 16-OTMS ester is completely separated in peak II. There was no evidence of any other mono OH ester that might be derived from hydroperoxides on C positions 8, 10, 15, and 17. The tri-OTMS derivatives with end substituents on C-9 (m/e 259) and C-12 (m/e 187) emerge in peak III, and with end substituents on C-13 (m/e 315) and C-16 (m/e 131) emerge in peak IV. These assignments were confirmed by mass chromatography showing a scan of the appropriate masses (Fig. 2). The identity of peak III was further confirmed by peak enhancement resulting when *erythro*-9,10,12-trihydroxyoctadecanoates were added to the sample. The small

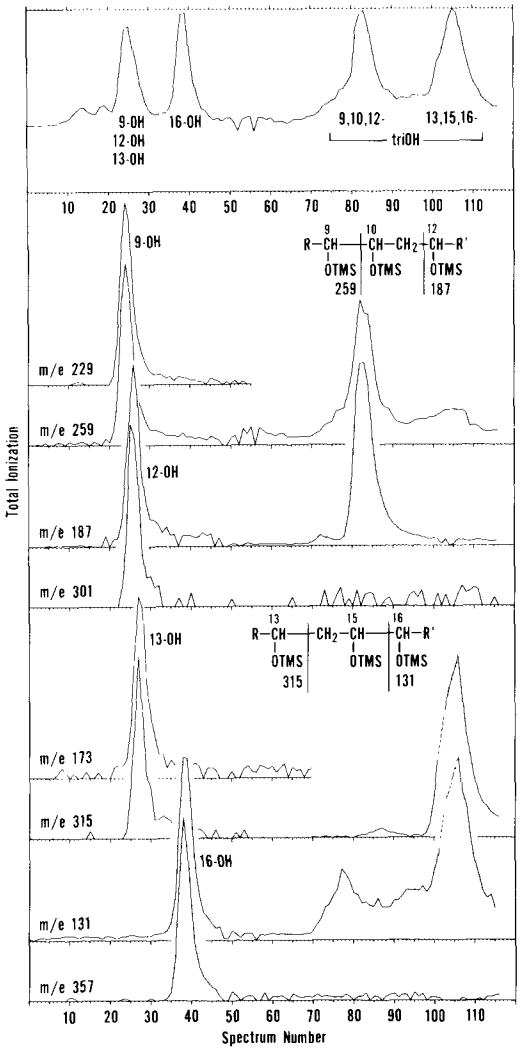


FIG. 2. Mass chromatography of hydrogenated-autoxidized methyl linolenate (peroxide value 1315).

shoulder of lower retention was also enhanced when the mixture of diastereoisomeric 9,10,12-trihydroxy esters was added to the sample. The MS of fractions from peak III obtained with those mixtures were identical to those of the original hydrogenated oxidized sample. These results are consistent with the presence of 9,10,12-triOTMS esters (peak III) and 13,15,16-triOTMS esters (peak IV).

Quantitative GC-MS analysis was carried out by the same computer summation method standardized in the preceding papers for autoxidized oleate and linoleate (1,2). The results obtained with artificial mixtures of synthetic 9-, 12-, 13-, and 16-hydroxyoctadecanoate were

TABLE II

Analysis of Synthetic Hydroxyoctadecanoates by GC-MS and by GC

Known mixtures	GC-MS (rel. %)[a]				GC (rel. %)[b]	
	9-OH	12-OH	13-OH	16-OH	9- + 12- + 13-OH	16-OH
Mixture 1	24.8	24.8	26.3	24.1	75.9	24.1
Found	23.0	25.1	26.9	25.0	76.2	23.8
Mixture 2	29.2	10.6	10.7	49.5	50.5	49.5
Found	27.6	11.6	13.0	47.8	50.5	49.5
Mixture 3	15.9	35.8	31.7	16.6	83.4	16.6
Found	14.7	36.5	32.6	16.2	83.4	16.6
Mixture 4	31.4	21.8	18.4	28.4	71.6	28.4
Found	28.6	24.0	18.9	28.5	71.5	28.5
Mixture 5	20.3	28.7	28.8	22.2	77.8	22.2
Found	18.4	29.6	29.8	22.2	78.7	21.3
Mean rel. % for 5 mixtures	24.3	24.3	23.2	28.2	71.8	28.2
Found	22.4	25.3	24.3	28.0	72.1	27.9
Standard deviation		0.63			0.28	

[a]Based on computer summation of masses 229+259 for 9-OH isomer, masses 187+301 for 12-OH isomer, masses 173+315 for 13-OH isomer, and masses 131-357 for 16-OH isomer (OTMS derivative).

[b]Based on separation shown in Figure 1C: Peak I = 9- + 12- + 13-OH isomer, Peak II = 16-OH isomer.

TABLE III

GC-MS Analysis[a] of Isomeric Hydroxyoctadecanoate from Autoxidized Methyl Linolenate

Peroxide value	Temp., C	Relative percent			
		9-OH	12-OH	13-OH	16-OH
134	25	28.1	11.4	11.4	49.1
495	25	31.7	10.7	10.9	46.7
1315	25	31.9	8.2	10.0	49.9
337	40	30.4	11.9	13.0	44.8
710	40	33.5	10.6	11.9	44.1
1183	40	35.1	10.0	12.2	42.7
212	60	26.6	10.3	11.1	52.0
639	60	33.4	10.9	12.5	43.4
1130	60	28.2	13.1	11.7	47.0
466	80	34.8	13.2	10.5	41.4
1839	80	33.5	8.9	12.5	45.1

[a]See footnote a in Table II.

quantitatively reliable as shown by a calculated standard deviation of 0.63 between known compositions and experimental values (Table II). A further check was made by quantitative GC analysis of the 16-hydroxy isomer which was separated from the other three hydroxy isomers (Fig. 1C). The standard deviation from the known mixtures calculated to be 0.28.

Samples of methyl linolenate oxidized to different peroxide values and at different temperatures show that the proportion of 9- and 16-hydroxy esters was significantly higher (75-82%) than the 12- and 13-hydroxy esters (18-25%) (Table III). In a previous paper (8), we estimated the hydroperoxides from linolenate autoxidized at 37 C by analyzing cleavage products of monoenes derived by dehydration from hydroxyoctadecanoate derivatives. The distribution obtained (30.2% 9-OH, 10.7% 12-OH, 9.8% 13-OH, 48.1% 16-OH) is in good agreement with out present GC-MS analyses in Table III.

The quantitative GC-MS method was also applied to the analysis of autoxidized mixtures of linolenate with linoleate and with oleate. Two different binary mixtures and one ternary

TABLE IV

GC-MS Analysis of Autoxidized Mixtures of Oleate (01):Linoleate (Lo):Linolenate (Ln)

Mixtures 01:Lo:Ln	PV	Temp. C	Relative percent							Hydroperoxides, %[a]		
			8-OH	9-OH	10-OH	11-OH	12-OH	13-OH	16-OH	01	Lo	Ln
0:1:1	200	60	---	36.3	---	---	3.9	43.2	16.6	---	64	36
	491	60	---	42.1	---	---	3.6	36.7	17.6	---	62	38
	1197	60	---	44.1	---	---	3.4	38.1	14.4	---	68	32
0:1:1	401	80	---	38.3	---	---	4;1	34.5	23.1	---	52	48
	1238	80	---	41.0	---	---	5.2	38.8	15.0	---	64	36
0:9:1	340	80	---	35.2	---	---	0.1	36.9	27.9	---	68	32
	1708	80	---	47.9	---	---	1.6	46.6	3.9	---	90	10
1:0:1	120	60	5.2	29.3	5.3	4.8	6.8	12.1	36.5	24	---	76
	574	60	6.0	27.3	5.7	4.5	6.8	8.4	41.3	15	---	85
	763	60	6.7	29.5	5.6	5.8	8.0	9.4	35.0	24	---	76
1:0:1	552	80	5.3	21.2	2.9	3.1	5.6	13.5	48.4	4	---	96
	1181	80	9.7	21.3	8.6	8.2	11.1	8.4	32.7	22	---	78
9:0:1	530	80	24.5	24.0	20.7	24.6	1.2	1.3	3.7	91	---	9
	1353	80	25.8	20.9	20.9	24.2	0.9	0.9	6.2	89	---	11
1:1:1	114	60	2.6	35.7	2.0	2.3	5.0	28.1	24.4	9	39	52
	563	60	3.1	41.0	2.1	2.3	3.6	32.5	15.4	9	57	34
	1071	60	3.3	41.4	2.2	2.3	3.1	33.5	14.2	9	60	31

[a]Assuming that 12-OH + 16-OH = 56.5% in autoxidized linolenate (Table III) and that 10-OH + 11-OH = 50% in autoxidized oleate (1). Ln-hydroperoxide = (12-OH + 16-OH) ÷ 0.565. 01-hydroperoxides = (10-OH + 11-OH) X2. Lo-hydroperoxides = 100 - (Ln-OOH + 01-OOH).

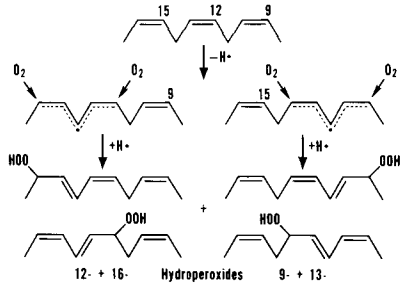


FIG. 3. Mechanism of linolenate autoxidation.

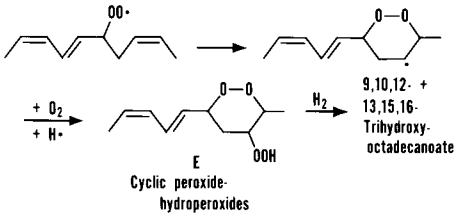


FIG. 4. 1,4-Cyclization of 12- and 13-hydroperoxides of linolenate.

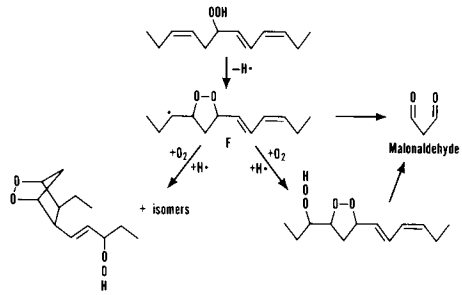


FIG. 5. Prostaglandin-like endoperoxides and malonaldehyde formation by 1,3-cyclization of 12- and 13-hydroperoxides of linolenate (14).

mixture were autoxidized at different levels, and the hydroxyoctadecanoate TMS derivatives were analyzed by GC-MS. With these mixtures, the 9-hydroxy ester comes from the hydroperoxides of all three fatty esters and the 13-hydroxy ester from both linoleate and linolenate hydroperoxides. The 8-, 10-, and 11-hydroxy esters come from oleate hydroperoxides whereas the 12- and 16-hydroxy esters come only from linolenate hydroperoxides. To estimate the different hydroperoxides in these autoxidized mixtures, one can use the values for the 12- and 16-hydroxy esters which are unique for the linolenate hydroperoxides, and the values for the 10- and 11-hydroxy esters which are unique for the oleate hydroperoxides. The different hydroperoxides were thus estimated in autoxidized mixtures by assuming that the sum of the 12- and 16-hydroxy esters are formed in the same proportion (averaging 56.5%) as in pure linolenate, and that the sum of the 10- and 11-hydroxy esters form 50% of the oleate hydroperoxides.

With equal binary mixtures of esters autoxidized at levels below 10% (PV less than 610), the proportion of linolenate hydroperoxides varied from 36 to 48% compared to 52 to 64% for linoleate hydroperoxides, and was much greater than that of oleate hydroperoxides (Table IV). At higher peroxide values, the proportion of linoleate hydroperoxides was even larger than that of linolenate hydroperoxides which in turn was larger than that of oleate hydroperoxides.

With the linoleate-linolenate mixture containing 10% linolenate autoxidized to a peroxide value of 340, 32% of the hydroperoxides originated from linolenate. At the more advanced levels of oxidation, as expected, the dominant hydroperoxide originates from the dominant fatty ester in the mixture.

With the oleate-linoleate-linolenate mixtures autoxidized to a PV of 114, 52% of the hydroperoxides were derived from linolenate and 39% from linoleate. This ratio is reversed at the higher peroxide levels. In all samples, the contribution from oleate hydroperoxides remained constant at 9%.

DISCUSSION

In previous papers (8,11), we formulated a mechanism for linolenate autoxidation based on that of methyl linoleate. Hydrogen abstraction on C-11 and C-14 of linolenate produces two pentadiene radicals, each having two equivalent sites for O_2 attack: C-9 and C-13 on one hand, and C-12 and C-16 on the other hand (Fig. 3). As with linoleate (11), the products expected by this scheme would be equal amounts of 9-, 13-, 12-, and 16-hydroperoxides with *cis,trans/trans,cis*-conjugated diene systems. However, our present GC-MS results show a significantly larger concentration of the 9- and 16-hydroperoxides than the 12- and 13-hydroperoxides. This distribution is in remarkably good agreement with our earlier results based on an entirely different chemical characterization scheme (8). We suggested at that time that either the pentadiene radicals prefer to react with O_2 at the end C-9 and C-16 positions, or that the 12- and 13-hydroperoxide isomers are more easily decomposed. Steric factors might indeed be invoked for greater attack of O_2 on C-9 and C-16 than on C-12 and C-13 on one hand, and greater attack on C-16 than on C-9 on the other hand.

Following our earlier work, Haverkamp Begemann et al. (12) identified isomeric cyclic

peroxide-hydroperoxides among the more polar products of linolenate autoxidation. The peroxides with a six-membered ring between C-9 and C-12 on one hand, and between C-13 and C-16 on the other hand, are derived from the corresponding 12- and 13-hydroperoxides of linolenate. Indeed, Gunstone (13) suggested that the reducted yield of 12- and 13-hydroperoxides we reported (8) may be due to their unique 1,5-diene structure leading to the formation of the six-membered cyclic peroxide-hydroperoxides (Fig. 4).

The reduced yields of 12- and 13-hydroperoxides from linolenate can also be explained by their tendency to cyclize into prostaglandin-like endoperoxides. Pryor et al. (14) reported evidence for endoperoxides from autoxidized linolenate with structures related to those of the endoperoxides formed from arachidonic acid in the biosynthetic pathway established for prostaglandin (15). They formulated the internal 12- and 13-hydroperoxides as the source of endoperoxides which on decomposition give malonaldehyde and a positive TBA test (Fig. 5). The chemistry of linolenate autoxidation becomes particularly important today if the suggestion of Pryor et al. (14) is valid that some of the symptoms of lipid peroxidation in vivo could originate from nonenzymatically produced prostaglandins or their stereoisomers.

The higher oxygenated products indicated in this paper by GC-MS in reduced or hydrogenated oxidized linolenate include trihydroxy esters. The 9,10,12- and 13,15,16-trihydroxy esters determined in this paper and identified by Haverkamp Begemann et al. (12) can be produced by hydrogenation from either the six-membered cyclic peroxide-hydroperoxide of type E or from the endoperoxyhydroperoxides of type F formulated by Pryor et al. (14).

In the previous paper of this series, we analyzed the hydroperoxides from different autoxidized mixtures of oleate and linoleate (2). Linoleate hydroperoxides dominated the products from different mixtures at low levels of oxidation. In the present study, with equal mixtures of esters autoxidized at levels below 10%, the proportion of linolenate hydroperoxides actually detected was less than that of linoleate but much greater than that of oleate hydroperoxides. These results reflect not only the greater ease of hydrogen abstraction from linolenate and linoleate compared with that of oleate, but also suggest the loss of linolenate hydroperoxides by cyclization and secondary oxidation. When the mixtures contained only 10% linolenate, the proportion of linolenate hydroperoxides was considerably greater. In mixtures containing the same initial concentration of linoleate and linolenate, the higher level found of linoleate hydroperoxide can be explained by the difference in relative rates of propagation and termination of the respective peroxy radicals. Although predominant autoxidation of linolenate is likely, linolenate hydroperoxides would be expected to decompose more readily and not accumulate to as high a level as the other hydroperoxides (11).

The GC-MS approach is thus useful in determining the origin of hydroperoxides formed in autoxidized mixtures of unsaturated fatty esters. The next paper in this series will deal with a study of oxidation products from mixtures of unsaturated fatty esters in vegetable oils to determine their relative role as precursors of off-flavors.

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Gas Chromatographic Separation of Triglycerides Based on Their Degree of Unsaturation

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ABSTRACT

Gas liquid chromatographic separation of long chain triglycerides based on their degree of unsaturation has been carried out on a polar siloxane, SILAR 10C. The equivalent chain lengths of triglycerides with 36-54 acyl carbons and 0-9 double bonds are presented. The resolution of triglycerides of coconut oil by combining argentation thin layer chromatography and gas chromatography on SILAR 10C is described.

INTRODUCTION

During the last 15 years, gas liquid chromatography (GLC) of triglycerides in natural fats and oils has progressed from an advanced technique in a limited number of laboratories to an indispensable tool for widespread laboratory study of fats and oils. In general, GLC analysis of triglycerides has been carried out on the basis of the total number of acyl carbons in their molecules using a regular type GLC instrument equipped with dual hydrogen flame detectors, automatic temperature programmer, and low-load nonpolar liquid phase columns as described in reviews (1-4). However, the effective separation of triglycerides based on their degree of unsaturation on a polar phase has not been reported although it would be useful to many lipid chemists. Under the conditions of the low liquid phase loading and high temperature required for the GLC of triglycerides, the conventional polyester columns have an extremely short life due to the rapid loss and decomposition of the liquid phase. In addition, a stable base line cannot be obtained due to the polyester bleeding and the high sensitivity required for the GLC of triglycerides. The polar siloxane columns, which possess moderate thermal stability, permit a partial resolution in the GLC of lower molecular weight triglycerides (5), but medium and high molecular weight triglycerides have not been resolved on the phases.

In our early work on GLC separation of wax esters (6,7) and fatty acid cholesteryl esters (8) on the liquid phase SILAR 10C, we observed that the appropriate column temperatures were unexpectedly as low as 270 C for the wax ester of carbon number 60 and the cholesteryl ester of carbon number 55. These facts were attributed to the low affinity of the esters having a nonpolar character for the polar phase. This result made us hopeful of achieving GLC separation of triglycerides based on their degree of unsaturation.

The resolution of triolein and tristearin based on the difference of three double bonds on a nonpolar methyl siloxane JXR column (1.8 m length) has been reported (3). In this study, a fair resolution of the peaks based on the difference of one double bond was acheived in the GLC on SILAR 10C of triglycerides up to trilinolenin (54:9) in natural fats and oils. A successful GLC of coconut oil triglycerides was performed as part of this study.

MATERIALS AND METHOD

Materials

Coconut oil used in this study was a refined commercial product. The triglyceride fraction was isolated from the oil by column chromatography of a 4 g sample with successive developments by hexane and ether - hexane (5:95, v/v) on 2.8 cm inside diameter column packed with 70 g silicic acid (Wakogel C-200). The packing for GLC and standard materials (trimyristin, tripalmitin, tristearin, and triarachidin) were purchased from Applied Science Laboratories, State College, PA. Saturated triglycerides (C_{40}-C_{58}) were obtained by hydrogenation of a sperm whale body oil. Partially purified references, triolein, trilinolein, and trilinolenin were isolated in our laboratory from olive, safflower, and linseed oils, respectively, by silver nitrate thin layer chromatography ($AgNO_3$--TLC).

Methods

GLC of triglycerides was carried out with a Shimadzu GC-6AM instrument equipped with dual hydrogen flame ionization detectors, an automatic temperature programmer, and U-shaped glass columns (0.5 m or 1.5 m length, 3mm inside diameter) packed with 100-120 mesh Gas Chrom Q coated with 3% or 5% (w/w) SILAR 10C. In this instrument, the end of the glass column is directly connected to the detector without an intermediate tube. These columns were used for a few months at 250 to

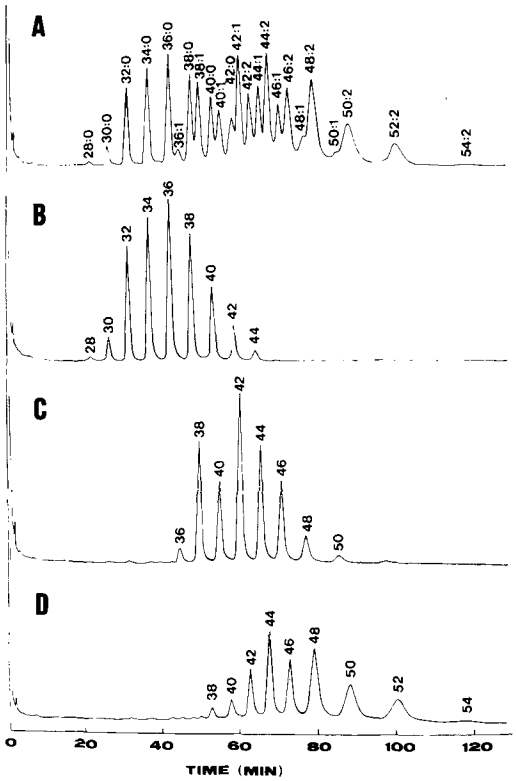

FIG. 1. Gas liquid chromatographic resolution of saturated (I), monoenoic (II), and dienoic (III) triglycerides on SILAR 10C. (A) Mixture of I, II, and III (about 30, 30, and 40%). (B) I. (C) II. (D) III. B, C, and D were separated from coconut oil by $AgNO_3$-TLC. Conditions: 1.5 m x 3 mm columns packed with 3% SILAR 10C on Gas Chrom Q (100-120 mesh), temperature programmed (180-270 C and 1 C/min). Injector and detector temperature: 335 C. Carrier gas: N_2, flow: 90 ml/min.

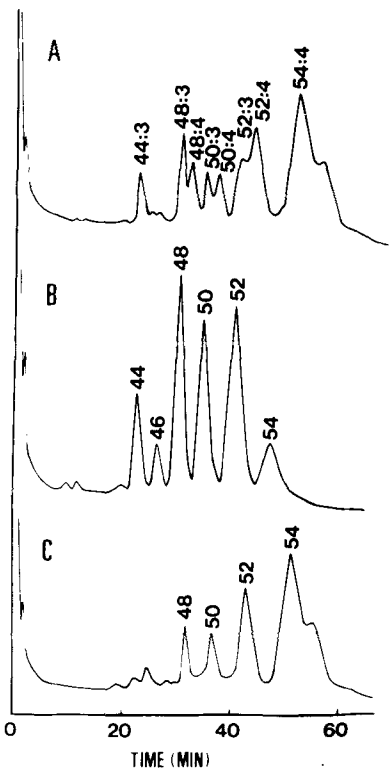

FIG. 2. Gas liquid chromatographic resolution of trienoic (I) and tetraenoic (II) triglycerides on SILAR 10C. (A) Mixture of I and II (about 30 and 70%). (B) I. (C) II. B and C were separated from coconut oil by $AgNO_3$-TLC. Conditions: 0.5 m x 3 mm columns packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh), temperature programmed (240-270 C at 1 C/min). Injector and detector temperature: 340 C. Carrier gas: N_2, flow: 80 ml/min.

270 C. The detector and an on-column injector were maintained at 340 C in the same heater block. Under the standard conditions, 0.4-0.6 μl of 0.25% (w/v) solution of triglyceride sample in hexane or chloroform was injected directly on the column packing, and at the same time, the temperature programming was started from 180 C at 1 C/min with a N_2 flow of 80 ml/min. The final temperature of 270 C was maintained until all peaks of triglycerides emerged. The attenuation was set in the region of 1/32 or 1/64 x 10^4. GLC analysis based on the carbon number from nonpolar columns was carried out with the same instrument equipped with 0.3 m columns packed with 80-100 mesh Chromosorb W coated with 2% (w/w) OV-17. The column was programmed from 180 to 335 C at 2 C/min with a N_2 flow rate of 90 ml/min. The sample size was 1 μl of 1% solution in hexane or chloroform.

Fatty acid compositions of coconut oil triglycerides were determined by GLC of methyl esters (9). The methyl esters were also analyzed on a Shimadzu 6AM instrument with a Chromatopac ElA computerized digital integrator. The glass columns (1.5 m x 3mm inside diameter) packed with 5% SILAR 10C on 100-120 mesh Gas Chrom Q were programmed from 65 to 220 C at 4 C/min, or isothermally at 195 C to separate 20:1 and 18:3. The N_2 flow rate was 30 ml/min.

The fractionation of triglycerides based on unsaturation was carried out by $AgNO_3$-TLC on silicic acid (Wakogel B-10) impregnated with 15% silver nitrate (w/w). The plates were developed twice with benzene-chloroform (9:1, v/v) for coconut and olive oils, twice with chloroform-methanol (97:3, v/v) for safflower oil, and twice with chloroform-methanol (95:5, v/v) for linseed oil. The bands were detected

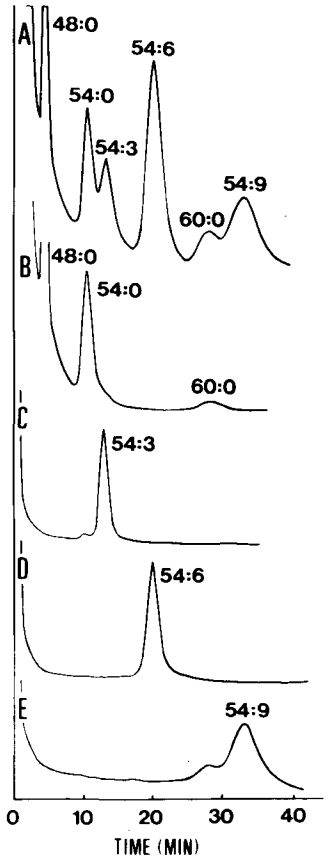

FIG. 3. Gas liquid chromatographic resolution of C54 triglycerides on SILAR 10C (A) Mixture of B, C, D, and E. (B) Saturated triglycerides. (C) Triolein. (D) Trilinolein. (E) Trilinolenin. C, D, and E were separated from olive, safflower, and linseed oils, respectively, by $AgNO_3$-TLC. Conditions: 0.5 m x 3 mm columns packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh). Column temperature: 270 C. Injector and detector temperature: 345 C. Carrier gas: N_2, flow: 150 ml/min.

under ultraviolet light after spraying with 2',7'-dichlorofluorescein reagent, and extracted with diethyl ether. The major fatty acids of the triolein, trilinolein, and trilinolenin fractions obtained were oleic acid (94.9 wt %), linoleic acid (99.1 wt %), and linolenic acid (88.9 wt %), respectively.

RESULTS AND DISCUSSION

In the GLC of triglycerides on a polar column, the highly temperature-stable cyanosiloxane SILAR 10C was found to be a suitable liquid phase. Other SILAR phases, probably because of their lower polarities, required higher elution temperatures for the emergence of C_{54} triglyceride peaks above the limit for

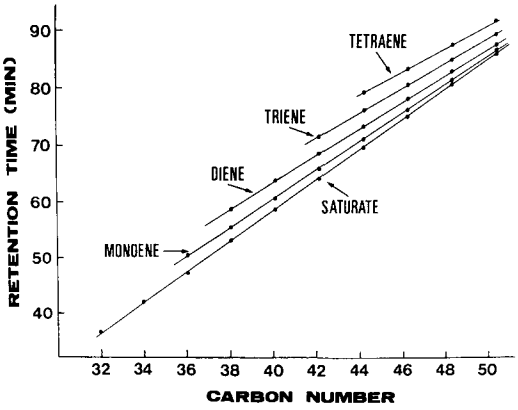

FIG. 4. Linear relationship between retention times and the number of acyl carbons of triglyceride homologues of different unsaturation. Conditions: 0.5 m x 3 mm columns packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh), temperature programmed 180-270 C at 1 C/min). Carrier gas: N_2, flow: 80 ml/min.

their decomposition. The other highly temperature-stable cyanosiloxane OV-275 showed sufficient polarity, but it did not have enough thermal stability for repeated analyses at 270 C under the conditions of our experiment.

Effect of the column length on the resolution of triglyceride peaks was investigated with the various columns. The results showed that a 3% SILAR 10C column of 1.5 m length and a 5% SILAR 10C column of 0.5 m length were effective for the resolution of triglycerides. To obtain satisfactory peak resolution and a sharp symmetrical peak pattern, the electrometer sensitivity was turned up as far as practical, until base line noise and base line drift could not be disregarded at 270 C, and the sample size was kept as small as possible.

Figure 1 shows the resolution of saturated, monoenoic, and dienoic triglycerides on 3% SILAR 10C (1.5 m length). To elute the peaks of C_{28} to C_{54} triglycerides with satisfactory resolution based on their degree of unsaturation, a flow rate of less than 90 ml/min carrier gas (N_2) was found to be desirable. This column showed better resolution than 5% SILAR column (0.5 m length) under the same condition. In GLC of the mixture of 38:0 and 38:1 (about 1:1, w/w), the former gave the 80% valley separation, while the latter gave only 60% under the same condition. However, the peaks of the unsaturated triglycerides containing more than 54 acyl carbons take too long to emerge on the chromatograms, no matter how high a nitrogen flow rate is applied.

Figure 2 shows the resolution of the trienoic and tetraenoic triglycerides on 5% SILAR 10C

TABLE I

Fatty Acid Compositions (mol %) of Coconut Oil Triglycerides[a]

Fatty acid	Degree of unsaturation of triglycerides[b]				
	Saturate	Monoene	Diene	Triene	Tetraene
6:0	0.33	0.26	—	—	—
8:0	10.45	7.58	3.77	1.70	0.88
10:0	7.89	4.14	2.33	1.01	0.49
12:0	54.11	30.96	14.20	6.81	3.32
14:0	17.65	11.04	10.21	6.13	3.01
16:0	7.26	9.57	14.67	10.92	7.63
16:1	—	0.13	0.40	0.68	1.60
18:0	2.30	1.29	1.87	1.11	0.92
18:1	—	34.66	36.73	40.62	28.17
18:2	—	—	15.45	29.75	49.63
18:3	—	—	—	—	1.13
20:1	—	0.20	0.20	0.40	0.34
22:1	—	0.18	0.18	0.88	2.89
Number of double bonds[c]	0.00	1.06	2.05	3.07	4.07

[a]GLC conditions: column 1.5 m length, 0.3 cm inside diameter packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh), temperature programmed (60-220 C at 4 C/min) and 195 C isothermally. Carrier gas: N_2, flow rate: 30 ml/min.

[b]Estimated from the position of the spot on $AgNO_3$-TLC plate. Recovery (wt %): saturate 82.2, monoene 10.4, diene 2.1, triene 2.5, and tetraene 2.8.

[c]Calculated from the fatty acid compositions.

TABLE II

Relative Retention Times (RRT) and Equivalent Chain Lengths (ECL) of Triglycerides on SILAR 10C Liquid Phase[a]

Triglyceride	RRT	ECL	Triglyceride	RRT	ECL
36:0	0.188	36.00	38:2	0.336	40.32
38:0	0.244	38.00	40:2	0.422	41.98
40:0	0.321	40.00	42:2	0.560	43.98
42:0	0.423	42.00	44:2	0.716	45.75
44:0	0.561	44.00	46:2	0.961	47.74
46:0	0.742	46.00	48:2	1.214	49.25
48:0	1.000	48.00	50:2	1.625	51.10
50:0	1.362	50.00	52:2	2.191	52.96
52:0	1.880	52.00	54:2	2.959	54.79
54:0	2.595	54.00			
56:0	3.613	56.00	42:3	0.674	45.32
58:0	5.090	58.00	44:3	0.852	46.92
60:0	5.472	60.00	46:3	1.090	48.55
			48:3	1.410	50.21
36:1	0.209	36.81	50:3	1.858	51.93
38:1	0.275	38.87	52:3	2.496	53.76
40:1	0.357	40.77	54:3	3.206	55.28
42:1	0.454	42.50			
44:1	0.604	44.53	48:4	1.665	51.24
46:1	0.804	46.54	50:4	2.178	52.79
48:1	1.084	48.52	52:4	2.852	54.57
50:1	1.471	50.49	54:4	3.689	56.12
52:1	1.982	52.33	54:6	4.841	57.70
54:1	2.702	54.25	54:9	7.906	60.32

[a]Coconut oil, hydrogenated sperm whale body oil, triolein, trilinolein, and trilinolenin were used to obtain these data. GLC conditions: 0.5 m x 3 mm columns packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh). Column temperature: 270 C. Carrier gas: N_2, 90 ml/min.

TABLE III

Comparison of Δ_tECL of Triglycerides with Those of Cholesteryl Esters, Wax Esters, and Methyl Esters[a]

Temperature	Triglyceride[b] 250-270[f]	Methyl ester[c] 150-190	Wax ester[d] 210-230	Cholesteryl ester[e] 250-260
Monoene	0.003	0.007	—	0.008
Diene	0.011	0.011	0.028	0.017
Triene	0.020	0.013	0.037	0.026
Tetraene	0.024	—	—	—

[a]The data c, d, and e were selected from our previous papers (7,8).
[b]C_{48} triglycerides.
[c]Oleate, linoleate, and linolenate.
[d]Cetyl linoleate and stearyl linolenate.
[e]Oleate, linoleate, and linolenate.
[f]250-270 Indicates that the Δ_tECL was calculated from ECL at 250 and 270 C.

TABLE IV

Composition of Coconut Oil Triglycerides by GLC on SILAR 10C (mol%)[a]

Carbon number	Degree of unsaturation of triglyceride				
	Saturate	Monoene	Diene	Triene	Tetraene
26	Trace	—	—	—	—
28	0.77	—	—	—	—
30	3.89	—	0.30	—	—
32	17.03	—	0.39	—	—
34	21.62	0.11	0.44	—	—
36	24.04	2.59	0.32	—	—
38	17.52	20.30	2.29	—	—
40	9.43	13.07	3.76	—	—
42	4.10	26.57	9.89	1.11	—
44	1.26	17.10	17.07	11.18	2.69
46	0.33	11.70	13.38	4.89	0.80
48	Trace	4.96	23.44	24.94	8.05
50	—	2.20	15.61	19.16	8.59
52	—	0.28	11.66	28.50	21.14
54	—	—	0.55	8.42	39.85
Others	—	1.12[b]	0.90[c]	1.81[d]	18.89[e]

[a]GLC conditions: column 0.5 m length, 0.3 cm inside diameter packed with 5% SILAR 10C on Gas Chrom Q (100-120 mesh), temperature programmed (180-270 C 1 C/min). Carrier gas: N_2, flow rate: 80 ml/min.
[b]30:0 0.34, 32:0 0.51, 34:0 0.27.
[c]34:1 0.42, 36:1 0.48.
[d]36:2 0.77, 38:2 1.04.
[e]42:3 0.48, 44:3 1.18, 48:3 0.11, 54:5 16.77, 54:6 0.35.

(0.5 m length). Each triglyceride has a characteristic retention time on the chromatograms depending on its carbon number and number of double bonds. Figure 3 shows the resolution of C_{54} triglycerides on the short column. The column permitted complete resolution of tristearin, triolein, trilinolein, and trilinolenin (eluting later than triarachidin). The carrier gas flow rate required was more than 130 ml/min to obtain sharp peaks and to finish the analysis in the appropriate time.

The peaks were identified by comparison of their retention times with those of each fraction separated by $AgNO_3$-TLC, and of their peak area ratios with those obtained by GLC on OV-17. The degree of unsaturation of the triglyceride fractions separated by $AgNO_3$-TLC was obtained from comparision of the position of the spot with those of the standards, and the number of double bonds was calculated from the fatty acid compositions of each fraction shown in Table I.

In the previous paper (8), the temperature for the emergence of cholesteryl esters in GLC on SILAR 10C phase was found to be about 20 degrees lower than on a nonpolar column. In this study, the most suitable column temperature for GLC of tristearin on SILAR 10C phase was about 40 degrees lower than that on a nonpolar column. The results show that the influence of polarity of the phase on the retention temperature is very significant in the GLC

TABLE V

Composition of Coconut Oil Triglycerides by GLC on OV-17 (mol %)[a]

Carbon number	Degree of unsaturation of triglyceride				
	Saturate	Monoene	Diene	Triene	Tetraene
26	Trace	—	—	—	—
28	0.40	—	—	—	—
30	3.44	0.14	0.07	—	—
32	17.22	0.25	0.36	—	—
34	21.84	0.39	0.50	—	—
36	24.10	2.44	1.19	0.65	—
38	17.64	19.99	3.33	0.92	—
40	9.65	12.78	3.95	Trace	—
42	4.23	26.79	9.92	1.18	1.08
44	1.22	17.31	16.96	10.30	4.58
46	0.28	12.03	12.98	4.43	1.24
48	Trace	5.27	22.65	24.24	8.65
50	—	2.35	15.13	20.07	8.41
52	—	0.26	12.35	28.47	19.13
54	—	—	0.62	9.75	56.90

[a]GLC conditions: column 0.3 m length, 0.3 cm inside diameter packed with 2% OV-17 on Chromosorb W (80-100 mesh), temperature programmed (180-335 C at 2 C/min). Carrier gas: N_2, flow rate: 80 ml/min.

TABLE VI

Calculation of Fatty Acid Carbon Recovery in Triglyceride Fractions[a]

Triglyceride fraction	TCN[b]	ECN[c]		RTC (%)[d]	
		SILAR 10C	OV-17	SILAR 10C	OV-17
Saturate	3655	3570	3576	97.7	97.8
Monoene	4320	4196	4208	97.1	97.4
Diene	4766	4641	4637	97.4	97.3
Triene	5073	4923	4939	97.0	97.4
Tetraene	5248	5224	5207	99.5	99.2

[a]Calculated from the data in Table I, IV, and V according to the method described by Kuksis et al. (12).
[b]TCN = theoretical carbon number.
[c]ECN = carbon number for experimentally determined triglyceride distribution.
[d]RTC = recovery of theoretical carbon yield.

of triglycerides. An explanation of this observation must include a large affinity of triglycerides with three long alkyl chains to a nonpolar phase.

According to the literature (1,10), the retention time of cholesteryl palmitate approaches that of saturated triglycerides of 43 acyl carbons on a nonpolar column. In this study, cholesteryl palmitate showed a retention time on SILAR 10C corresponding to a saturated triglyceride with 42 acyl carbons.

Figure 4 shows the linear relations in $\log_{10}$ retention time against carbon number in a homologous series of different unsaturation obtained by temperature-programmed GLC. The straight lines are not parallel, and the distance between the lines progressively decreases with increasing carbon number, in contrast with other fatty acid derivatives such as methyl esters, wax esters (7), and diglycerides (11). Possibly, this is due to a lesser polarity of unsaturated high molecular weight triglycerides in comparison with unsaturated low molecular weight triglycerides. Under isothermal conditions, the linear relationships exist only over a limited range of carbon numbers (ca. 6 carbons), in contrast to the results under conditions of temperature programming shown in Figure 4. Therefore, the equivalent chain lengths (ECL) of unsaturated triglycerides were calculated from the logarithmic retention times of neighboring saturated homologous pairs.

Table II lists the relative retention times (RRT) and ECL of triglycerides on SILAR 10C. The data show the progressive increase of the separation factors between the two neighboring homologues with increasing molecular weight in each fraction. On the other hand, the reverse relationship has been reported in GLC of triglycerides on nonpolar columns (1). In the GLC of

triglycerides on SILAR 10C, one double bond approximately corresponds to one acyl carbon in ECL. Therefore, dienoic triglyceride peaks are almost overlapped by the saturated triglycerides having two more carbons. Analogous separation patterns have been observed in the GLC of wax esters on the same phase (6,7). To resolve the critical pairs, a preliminary separation by another method, $AgNO_3$-TLC as described in this paper, or preparative GLC on a nonpolar column, is neccessary. No resolution due to the difference of a double bond position has been observed in this study, although it was reported in the GLC of wax esters in our previous papers (6,7).

In previous papers (7,8), it was pointed out that the ECL values of unsaturated wax esters and fatty acid cholesteryl esters were significantly dependent upon the column temperature in GLC on SILAR 10C. The effect has been represented by Δ_tECL, an increase of ECL for one degree raising of column temperature. In the GLC of triglycerides in this study, the effect was less obvious. Table III compares Δ_tECL for selected triglycerides, methyl esters, wax esters, and cholesteryl esters. The results show that Δ_tECL values of unsaturated triglycerides on SILAR 10C are less than those of wax and cholesteryl esters and are close to those of methyl esters. This fact suggests that ECL values of triglycerides on SILAR 10C are less sensitive to the effect of column temperature.

Tables IV and V compare the compositions of coconut oil triglycerides obtained by GLC on SILAR 10C and OV-17 columns, respectively. The compositions were calculated from the peak area ratios obtained by the GLC of each fraction isolated by $AgNO_3$-TLC. Table VI gives the recovery of the triglycerides on both polar and nonpolar columns. The good agreement indicates the reliability of the GLC on SILAR 10C for quantitative analysis of triglycerides.

Bezard et al. (13) have calculated the composition of 79 types of coconut oil triglycerides based only on the carbon number by GLC on a nonpolar phase JXR and GLC of the fatty acid butyl esters of the triglyceride fracitons obtained by preparative GLC on the same phase. The method presented in this paper provides detailed composition of the triglycerides based on the carbon number and number of double bonds.

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p-Chlorophenoxyisobutyrate Enhanced Retention of Homologous Lipoproteins by Human Aortic Smooth Muscle Cells

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ABSTRACT

Human aortic smooth muscle cells (SMC) specifically bind and take up indiscriminately both the lipid and protein moieties of homologous ^{125}I-very low density lipoproteins (VLDL) and ^{125}I-low density lipoproteins LDL). Sixty-five to 80% of absorbed lipids are incorporated into the cell lipids, preferentially into the phospholipid fraction. Twenty to 35% of the lipid bound and the protein moiety are eliminated from the cells. Half of the eliminated protein label is recovered as TCA soluble products. Five mM of p-chlorophenoxyisobutyrate (CPIB) raise the level of intracellular radioactivity derived from the lipid moieties of VLDL and LDL by about 40% via a reduced elimination. The processing of the protein moiety and lipoprotein binding to the cell surface are not affected by 5.0 mM of CPIB. CPIB lowers the incorporation of ^{14}C-acetate, ^{14}C-pyruvate, and ^{32}phosphate radioactivity into fatty acids and phospholipids of aortic SMC. Five mM of CPIB reduce the overall palmitic acid synthesis by shifting from de novo synthesis to the mechanism of chain elongation, although the further elongation to saturated C_{18}-C_{24} fatty acids is also depressed. The CPIB-enhanced retention of the lipid-derived lipoprotein radioactivity is interpreted as a compensatory mechanism providing cellular fatty acids which are deficient as a result of the CPIB inhibited synthetic processes.

INTRODUCTION

p-Chlorophenoxyisobutyrate (CPIB) and its ethyl ester (clofibrate) have been shown to interfere with hepatic fatty acid and sterol synthesis in rat and primates by direct or indirect action at diverse loci (1-8). This effect is assumed to be primarily responsible for the lowering of plasma lipid levels and for preventing the progressive lipid accumulation of the arterial wall in arteriosclerosis. However, despite the extensive clinical use of CPIB as an antiatherosclerotic agent, its effect on the arterial tissue itself is still unknown.

Arterial tissue has been shown to metabolize ^{14}C-acetate and ^{14}C-fatty acids and to incorporate the label into total lipids and their subfractions (9), but the plasma lipoproteins, which can easily traverse the aortic endothelium (10), are thought to be the main physiological lipid substrates of the arterial tissue. Since even human cultured arterial smooth muscle cells (SMC) are known to bind and rapidly take up lipoproteins from the culture medium (11), an investigation of the effect of CPIB on the lipoprotein metabolism of human aortic SMC should give further information on the action mechanism of CPIB, its tissue specificity, and its alleged protection of the arterial tissue against a pathological deposition of lipids.

This paper reports that CPIB enhances the retention of lipid radioactivity from homologous ^{125}I-low density lipoproteins (LDL) and ^{125}I-very low density lipoproteins (VLDL) by human arterial SMC through an inhibition of lipid release.

Other inhibitory effects are a depression of the de novo synthesis of fatty acids from ^{14}C-acetate and ^{14}C-pyruvate comparable to that described for hepatocytes and adipocytes and a reduction of the microsomal chain elongation of fatty acids.

MATERIALS AND METHODS

All chemicals and organic solvents were of analytical grade and were obtained from E. Merck (Darmstadt). Cow milk lactoperoxidase (160 U/mg protein) was purchased from Boehringer (Mannheim). Na [1-^{14}C] acetate (40 mCi/mmol), [U-^{14}C] pyruvate (15 mCi/mmol), [U-^{14}C] glycerol (20 mCi/mmol), [1-^{14}C] palmitate (50 mCi/mmol), carrier free ^{32}orthophosphate, and ^{125}Iodine were products of the Radiochemical Centre Amersham (U.K.). The sodium salt of p-chlorophenoxyisobutyric acid was a gift from Imperial Chemical Industries, Ltd., U.K.

Isolation and Iodination of Lipoproteins

Very low and low density lipoproteins (VLDL and LDL) were isolated from normal human plasma by sequential flotation in a Beckman preparative ultracentrifuge (L 2 65 B) according to Hatch and Lees (12). Lipoproteins

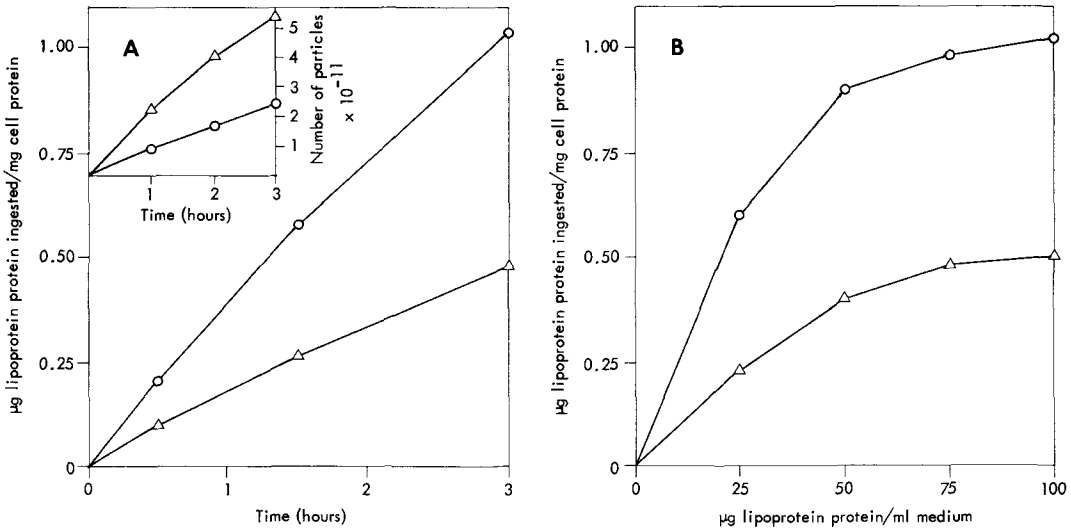

FIG. 1. Uptake of homologous ^{125}I-VLDL (○) and ^{125}I-LDL (△) as a function of time (A) and lipoprotein concentration (B) (3 hr experiment) by human aortic smooth muscle cells as expressed as μg internalized apolipoprotein/mg cell protein. The portion of absorbed protein is calculated from the specific radioactivity of ^{125}I-VLDL (21.9 x 10^3 cpm/μg lipoprotein protein) and ^{125}I-LDL (45.9 x 10^3 cpm/mg lipoprotein protein), taking in account the ratio of lipid/protein label (see Table I). Uptake refers to the radioactivity remaining in the cells after trypsinization and subsequent washes. All values are corrected for equal specific radioactivities of VLDL and LDL. The inset A gives the rates of VLDL and LDL uptake as expressed as number of ingested particles/hour.

were checked for purity by immunological methods. About 95% of the ^{125}I-radioactivity was precipitable with rabbit anti-LDL or anti-VLDL sera. Contamination of the lipoprotein preparations with albumin was excluded, since the rabbit antisera produced by our lipoprotein preparations did not react with human serum albumin. Labeling of lipoprotein was achieved with ^{125}Iodine by the lactoperoxidase method (13) which was found to be more protective and to deliver a higher labeling of the lipid moiety than the chloramine T method. Iodination of LDL and VLDL lipoproteins resulted in the specific labeling of both protein (apolipoprotein) and lipid moiety. For specific radioactivities, see Figure 1. The ratio of protein/lipid radioactivity was 1.3:1 (VLDL) and 1.4:1 (LDL). The ^{125}I-lipoproteins were dialyzed against 2 liters of 0.155 M NaCl containing 5 mM KI and two changes of 0.155 M NaCl for at least 48 hr at 4 C and then filtered through a Millipore Swinex 0.45 μm filter. Each isolated lipoprotein fraction migrated as a homogenous peak in agarose electrophoresis according to Kahlke (14). Ninety to 94% of the lipoprotein radioactivity was precipitated with 15% TCA at 4 C. After extraction of the precipitate with chloroform-methanol (2:1) and washing according to Folch et al. (15) and with 20 mM aqueous KI, 42-44% of the radioactivity was recovered in the chloroform-methanol phase.

Cell Cultures

The SMC used in the experiments were derived from the intimal-medial explant of the aorta thoracica of a 43-year-old patient. In agreement with Jarmolych et al. (16), arterial smooth muscle cells first aquire a fibroblast-like appearance in culture (17), but after a few weeks return to the appearance of smooth muscle cells. They were characterized by their typical content of numerous myofilaments, dense bodies, and pinocytotic vesicles, and by their tendency to grow in multilayers with hills and valleys. The human aortic smooth muscle cells differ from human skin fibroblasts in a longer lag period before outgrowing and a slower growth rate. Cells were cultivated in Eagle's minimum essential medium containing Eagle's salts (Serva) according to Kresse et al. (17), and modified by reduction of the $NaHCO_3$ concentration to 1.6 g/liter. The medium was supplemented with nonessential amino acids, 15% fetal calf serum (Serva), and penicillin-streptomycin (100 U of each/ml medium). Cells were grown to confluency in 75 cm^2 plastic flasks (Greiner) and were used after four to ten passages.

EXPERIMENTAL PROCEDURES

Cells grown to confluency were washed once

with Hank's solution after which 10 ml of fresh medium were added. The medium contained 20 μg/ml or the specified amount of ^{125}I-lipoproteins as expressed on a per protein basis, 1-10 μCi/ml of a precursor of lipid synthesis (see Table III), and the sodium salt of CPIB. The concentration of CPIB (5 mM) was according to previous studies (1,3,7) or adapted to the therapeutically achieved serum levels (0.5-1.0 mM). The mixtures were sterilized by filtration. The cells were incubated at 37 C for the time specified under a gas phase of 95% air/5% CO_2. For comparative experiments, identical subcultures of the same parent culture were used.

Binding of Lipoproteins to the Cell Surface

At the end of the incubation period, the medium was removed, and the cell monolayers were washed six times with 5 ml of Hank's solution containing 0.1% bovine serum albumin. The cells were removed from the flasks by trypsinization and then centrifuged. Two ml of the cell-free supernatant were added to 0.5 ml of 50% trichloroacetic acid, and after incubation at 4 C for 2 hr the precipitate was removed by centrifugation. Aliquots of the protein-free supernatant were counted in a gamma counter. Lipids were extracted from the precipitate with 3 ml of chloroform-methanol (2:1, v/v), which was removed with a nitrogen stream prior to counting radioactivity. Cells were disrupted by adding 1 ml of water followed by seven freeze-thaw cycles. Aliquots of the clear solution were removed for radioactivity counting and protein determination. The remainder was precipitated with TCA and centrifuged. To 1 ml of the TCA supernatant solution were added 0.01 ml of 40% solution of KI and 0.05 ml of a 30% solution of H_2O_2. After 5 min, the mixture was extracted with two volumes of chloroform to remove free iodine according to Bierman et al. (21). After phase separation, the radioactivity was counted in the water phase. Lipids were extracted from the TCA precipitate by adding 3 ml of chloroform-methanol (2:1, v/v). Lipid extracts were washed twice with 20.0 mM aqueous KI solution and analyzed as previously described (18).

Pulse Chase Experiments

In pulse chase experiments, the medium containing the labeled lipoproteins was removed after 3 hr, and the cells were washed six times with 5 ml of sterilized Hank's solution containing 0.1% BSA and then trypsinized, thus avoiding release and/or uptake of surface bound radioactivity during the chase. Cell pellets were resuspended in 10 ml of fresh incubation medium containing 200 μg/ml of nonlabeled lipoproteins and 5 mM CPIB. After incubating the cells for 3 hr longer the medium was removed, and an aliquot was counted to assess the released radioactivity. The cells were analyzed for lipid-protein- and TCA-soluble radioactivity (see below).

Determination of ^{14}C-CO_2

For determining the formation of ^{14}C-CO_2 from ^{14}C-pyruvate and ^{14}C-palmitate, 3 ml of incubation medium were placed in culture flasks sealed with rubber stoppers in which narrow glass tubes containing 0.3 ml of hyamine hydroxide were placed. Then 0.3 ml of 10 N NCl were injected for releasing CO_2 from the medium, and the flasks were kept for 1 hr at room temperature. The hyamine-containing glass tubes were removed, their outsides rinsed several times with water, and the tubes then transferred to plastic vials containing scintillation mixture for radioactivity measurement.

Isolation and Separation of Labeled Lipids

Total ^{125}I-lipids were extracted from cells, lipoproteins, and incubation medium with chloroform-methanol and washed with 20 mM aqueous KI. ^{14}C-lipids were extracted and washed according to Folch et al. (15). Separation into the major lipid subfractions was achieved on Silica Gel G plates using a solvent system of hexane-diethyl ether-acetic acid (75:25:1, v/v).

The spots were visualized by spraying the plates with 2% dichlorofluoresceine in ethanol, then scraped off, eluted with chloroform-methanol, and counted after removal of the organic solvent. For gas chromatography, total lipids were methylated with 5% sulfuric acid in absolute methanol, extracted with hexane, separated from unsaponifiable material by thin layer chromatography, and then analyzed in a Packard gas chromatograph with a 150 x 0.3 cm glass column packed with 15% DEGS on Gas Chrom P. The effluent was split in a 7:3 ratio, and the fatty acid methyl esters were collected in glass vials containing chilled chloroform. Isolated palmitic acid was decarboxylated according to Aronsson and Gürtler (19).

Radioactivity Measurements

^{125}I-radioactivity was counted in a LKB Wallace 80 000 counter; ^{14}C-radioactivity was measured after addition of 10 ml of toluene-ethylene glycol monomethyl ether (1:1, v/v) containing 0.21% PPO and 0.0026% POPOP in a Packard Tricarb Spectrometer (Model 3390). Quench corrections were made by internal standards.

TABLE I

Surface Binding, Uptake, and Regurgitation of Homologous ^{131}I-labeled VLDL and LDL by Human Aortic SMC in the Course of a 3 hr Pulse Followed by a 3 hr Chase in the Absence (A) or the Presence (B) of 5 mM CPIB

	cpm x 10^{-6}/mg Cell protein after 3 hr pulse				cpm x 10^{-6}in Medium/mg cell protein after 3 hr chase[a]	
	Surface binding[b]		Uptake[b]		Release[c]	
	A	B	A	B	A	B
VLDL[d]						
Lipids	20.7 ± 1.4	23.1 ± 1.4	26.2 ± 2.0	45.2 ± 2.9[e]	10.0 ± 0.6	5.8 ± 0.4[e]
Protein	21.1 ± 1.4	21.3 ± 1.5	17.9 ± 1.3	18.5 ± 1.2	8.1 ± 0.5	9.7 ± 0.7
TCA-solub. products	1.3 ± 0.1	1.8 ± 0.1	1.90 ± 0.1	2.0 ± 0.1	10.6 ± 0.7	10.6 ± 0.8
LDL						
Lipids	8.2 ± 0.5	11.0 ± 0.6	11.3 ± 0.7	22.6 ± 1.4[e]	3.1 ± 0.2	2.0 ± 0.1[e]
Protein	9.9 ± 0.5	10.6 ± 0.6	10.4 ± 0.6	10.9 ± 0.7	4.5 ± 0.3	4.5 ± 0.3
TCA-solub. products	0.95 ± 0.05	0.9 ± 0.05	0.9 ± 0.05	2.9 ± 0.2	6.2 ± 0.4	7.3 ± 0.4

[a]Chase experiments were performed after trypsinization and washing of aortic cells exposed to VLDL or LDL.

[b]The radioactivity remaining in the cells after trypsinization and two subsequent washes was considered as uptake while the label released by trypsin represented binding of lipoprotein to the cell surface.

[c]Data given for the CPIB effect on release refer to cells preincubated in the absence of CPIB. Statistically significant differences were examined by means of the t-test with reference to control experiment (absence of CPIB).

[d]Values are corrected for equal specific radioactivity of VLDL and LDL.

[e]p A/B <0.01.

TABLE II

Effect of CPIB on Distribution of Lipid Bound ^{125}I-radioactivity of LDL Used for the Incubation of Cellular Lipid Subfractions and to the Lipids Regurgitated into the Medium

Lipid[a] subfraction	Percent of total radioactivity in				
	LDL before incubation	(control)		5 mM CPIB	
		Cells	Chase medium	Cells	Chase medium
Phospholipids	22.3	47.6	25.2	51.3	22.4
Triglycerides	59.0	12.5	25.4	14.4	26.1
Total cholesterol + Diglycerides	12.1	25.7	36.3	23.4	39.0
Free fatty acids	7.7	14.1	13.1	10.6	12.4

[a]Total lipids of aortic cells and medium were obtained after a pulse-chase experiment (see Table I) and separated by preparative thin layer chromatography.

RESULTS

Lipoprotein Metabolism of Cultured Human Aortic SMC

The following metabolic features of lipoproteins as influenced by CPIB were investigated: surface binding of lipoproteins by the arterial SMC, uptake by the cells (absorption), and release of the absorbed lipoprotein components in an undegraded or partly degraded form.

The known interaction of lipoproteins with specific receptor sites of the cell membrane (20) results in a surface binding of lipoproteins. Trypsin treatment according to Bierman et al. (21) was used to distinguish between surface binding and absorption of lipoproteins after exposure of the cells to VLDL or LDL. Under the conditions used, the trypsinized cells retain their full viability, and no leakage of cytoplasmatic compounds, as judged by lactate dehydrogenase activity measurements, was detectable. After a 3 hr incubation period and extensive washing of the cells, 40-45% of the total ^{125}I-radioactivity associated with the arterial SMC are trypsin-removable and are considered to be surface-bound to the cell membrane receptors (Table I). The surface binding of LDL and VLDL is not influenced by CPIB.

Iodinated LDL and VLDL are taken up by aortic SMC at different rates for up to 3 hr. From the data of Figure 1, it appears that the VLDL uptake is more rapid than that of LDL (1A). The uptake follows saturation kinetics (1B) suggesting a process similar to adsorptive endocytosis.

The uptake rates given in Figure 1 are expressed as μg incorporated lipoprotein protein/mg cell protein, but comprising the total (lipid- and protein-bound) radioactivity of VLDL and LDL. However, if the uptake is calculated (inset of Fig. 1) for the number of particles based on the average molecular weights (VLDL = 25 x 10^6, LDL = 2.5 x 10^6) (22), then at equal particle concentration the LDL uptake will exceed that of VLDL. These results correspond to previously described data of Albers and Bierman (23).

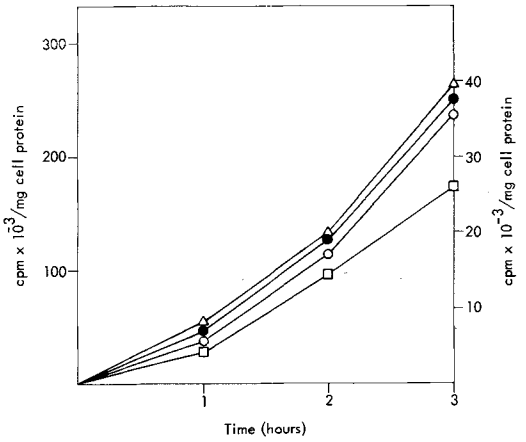

FIG. 2. Time course of incorporation of [1-^{14}C]acetate (○), [1-^{14}C]palmitate (△) (left ordinate), [U-^{14}C]glycerol (●), and [^{32}P]phosphate (□) (right ordinate) radioactivity into total lipids of aortic SMC.

The influence of CPIB on the net uptake of lipoproteins is evident from the data of Table I. Five mM of CPIB elevates the intracellular VLDL and LDL radioactivity by about 40 and 60% ($p < 0.001$), respectively, but the increased amount of radioactivity is almost totally due to the lipid-bound label which increases by about 100% as compared with the control cells. The balance of the apolipoprotein component shows only minor variations under the influence of CPIB.

After uptake of the lipoproteins, the lipids are, in part, subjected to cellular lipid metabolism being distributed between the individual

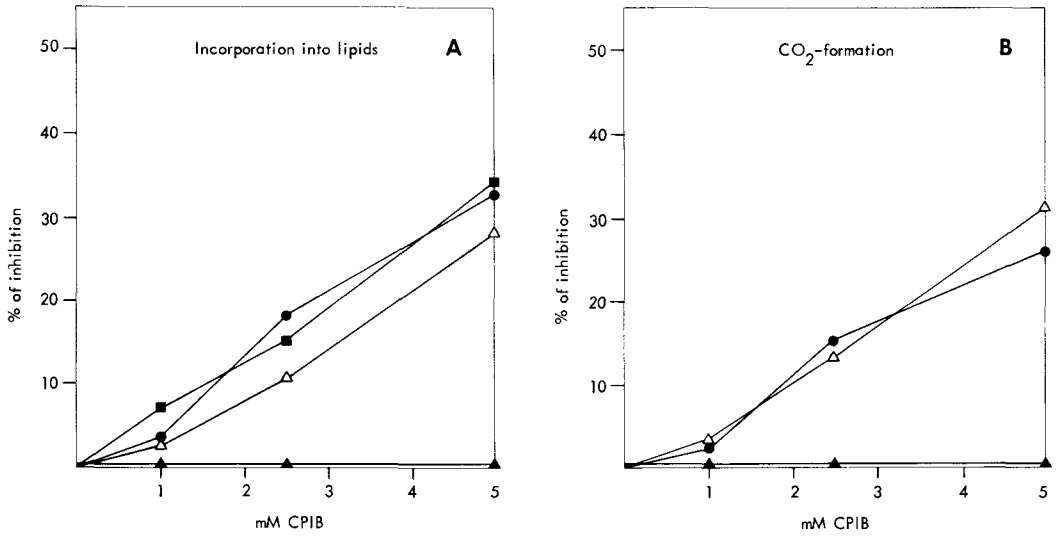

FIG. 3. Effect of CPIB on ^{14}C-acetate (○), ^{14}C-pyruvate (△), and ^{32}P-phosphate (□) incorporation into total lipids (A) and ^{14}C-CO_2 formation (B) by aortic SMC. The incubations contained 8-10 x 10^6 cells and 1.0 mM acetate, pyruvate or phosphate. CPIB was added at the indicated concentrations. The reactions were carried out for 3 hr.

lipid subfractions. The radioactivity pattern of the cellular ^{125}I-lipids, however, differs from that of the VLDL and LDL added to the medium. From the data of Table II, it appears that this difference is the result of transacylation processes of ^{125}I-acyl groups from incorporated triglycerides to phospholipids and cholesterol, leading to a preferential labeling of the cellular phospholipids.

About 25% of the lipid-bound label is released from the cells into the medium. In this fraction, the radioactivity is equally distributed between phospholipids, triglycerides, and cholesterol. Its radioactivity distribution reflects neither the radioactivity pattern of cellular lipids nor that of the fed ^{125}I-lipoprotein, suggesting that the process of elimination is the result of a selective export process.

During incubation, a small portion of the labeled compounds underwent deiodination which accounted for 2-10% of degraded lipoprotein radioactivity. No significant differences could be evaluated in the deiodination rates of ^{125}I-LDL or ^{125}I-VLDL. All data were corrected for the presence of free iodine (see Methods).

Five mM of CPIB depress the release of lipoprotein lipids from the cells by about 30% (LDL) and 40% (VLDL) (Table I), but neither the distribution pattern of labeled cellular lipids nor that of the relased lipids are affected (Table II).

In contrast to the incorporated lipids, the lipoprotein-protein label is quantitatively released into the medium during the chase period, half of the label being recovered as TCA soluble products. There were, however, no significant differences in the release of labeled protein and TCA soluble material between the control and CPIB-treated cells (Table I).

The faster elimination of protein-bound radioactivity rather than of lipid-bound radioactivity from the SMC explains the lowering of the ratio of the intracellular protein/lipid label (0.8 and 1.0) when compared with the corresponding ratio of the fed ^{125}I-lipoprotein (1.3 and 1.4).

Fatty Acid and Lipid Biosynthesis

Fatty acids, pyruvate, glycerol, and phosphate are all potential substrates for lipid synthesis in arterial wall cells. Their uptake by SMC (Fig. 2), incorporation into cellular lipids, and oxidation to CO_2 was linear over a period of 3 hr. The utilized radioactivity is partly incorporated into lipids, partly oxidized to $^{14}CO_2$, or metabolized by other (not identified) pathways. CPIB effects a dose-dependent depressing of the incorporation of ^{14}C-acetate and ^{14}C-pyruvate and of ^{32}p-phosphate into the cellular lipids while the labeling by ^{14}C-glycerol is unaffected. Fractionation of total lipids revealed that labeling of phospholipids was depressed to a greater extent than triglycerides.

The fact that the CO_2-formation from

TABLE III

Utilization of Labeled Metabolic Precursors by Aortic SMC Incorporation of Their Radioactivity into Total Lipids and Oxidation to CO_2 in Control (A) and CPIB Treated Cells (B) After a 3 hr Incubation Period

		nmole/mg Cell protein[a]					
		Utilized by aortic fibroblasts		Recovered [^{14}C] CO_2		Incorporated into total lipids	
[1-^{14}C] acetate	A	5.98 ± 0.42	100			3.28 ± 0.32	100
	B	5.00 ± 0.33	84			2.21 ± 0.18	68[c]
[U-^{14}C] pyruvate	A	0.38 ± 0.026	100	0.013 ± 0.002	100	0.05 ± 0.006	100
	B	0.27 ± 0.017	86	0.010 ± 0.001	70[c]	0.04 ± 0.004	72[c]
[U-^{14}C] glycerol	A	5.60 ± 0.59	100	n.d.		1.00 ± 0.11	100
	B	5.30 ± 0.55	94	n.d.		1.00 ± 0.10	99
[1-^{14}C] palmitate	A	54.52 ± 5.7	100	1.64 ± 0.25	100	39.0 ± 3.3	100
	B	57.36 ± 6.0	105	1.74 ± 0.28	106	42.1 ± 4.2	108
[^{32}P] phosphate	A	186.3 ± 20.0[e]	100	- -		26.1 ± 3.0	100
	B	138.6 ± 11.9[e]	74	- -		17.5 ± 1.9	67[d]

[a]Radioactivities added to the medium (as expressed as nmole/mg cell protein) were 195 (^{14}C-acetate), 1200 (^{14}C-glycerol), 19.0 ^{14}C-palmitate), and 3900 (^{32}phosphate).

[b]All data are expressed as means and standard deviation of five experiments.

[c]$p<0.01$.

[d]$p<0.02$.

[e]dpm x 10^3.

TABLE IV

^{14}C-Acetate Radioactivity Incorporated into Palmitic Acid and Percentage of Radioactivity Present in the Carboxylic Group of Palmitic Acid Given as Ratio Carboxylic Group/Acetyl Unit

	^{14}C-Radioactivity		
	dpm x 10^{-3}/mg Cell protein incorporated into		
CPIB-addition	Total fatty acids[a]	Palmitic acid	Radioactivity carboxylic group/acetyl unit
None	240 ± 18	107 ± 10	1.0 ± 0.05
5 mM	164 ± 11	37 ± 3	1.3 ± 0.04

[a]Total fatty acids were prepared from total lipids following incubation of aortic SMC for 3 hr with [1-^{14}C]acetic acid (pulse) and in a acetic acid free medium for further 3 hr in the presence or absence of CPIB (chase).

^{14}C-pyruvate is inhibited to the same extent as the incorporation of this precursor into the lipids, but that the oxidative degradation of palmitic acid to CO_2 remains unaffected, suggests a specific block of pyruvate oxidation. The results of two series of experiments listed in Tables IV and V support this assumption.

From the radioactivity incorporated into palmitic acid (isolated from total cellular lipids) after incubation with ^{14}C-acetate and from the radioactivity ratio of the carboxyl group vs. the acetyl units of the alkyl residue, it is evident that CPIB reduces the synthesis of palmitic acid from ^{14}C-acetate, and that in the presence of CPIB the mechanism shifts from de novo synthesis to chain elongation (Table IV).

Since the chain elongation of preformed C_8-C_{12} acyl units to C_{16}-palmitic acid is considered to proceed via the mitochondrial elongation system, the elongation of ^{14}C-palmitic acid by the microsomal chain elongation system, which is assumed to be responsible for the formation of higher saturated fatty acids from palmitate, was also investigated. In the control, 73% of the ^{14}C-palmitic acid was elongated to 18:0-24:0 fatty acids, but CPIB reduced the portion of palmitate which is elongated by the microsomal system to 35%, with the bulk of the ^{14}C-palmitic acid being incorporated into lipids without further metabolism (Table V).

TABLE V

Chain Elongation of [1-^{14}C]Palmitic Acid as Influenced by 5 mM CPIB

CPIB	dpm/Total saturated fatty acids[a] (percentage)				
	16:0	18:0	20:0	22:0	24:0
None	7100 (26%)	12500 (45.6%)	6600 (24%)	680 (2.5%)	530 (1.9%)
5 mM	22000 (65%)	6800 (20%)	4000 (11.8%)	630 (1.9%)	430 (1.3%)

[a]The saturated fatty acids of total lipids were isolated by removing the unsaturated fatty acids by argentation thin layer chromatography, transformed to methyl ester and submitted to gas liquid chromatography.

DISCUSSION

The CPIB and clofibrate-induced alterations of lipid and energy metabolism of liver (1,2,6), adipose tissue (24,25), and cells derived from these organs (1,3,7) are considered to be largely responsible for the hypolipidemic effect of CPIB and are thought to protect the arterial tissue against lipid accumulation. Although CPIB is in extensive clinical use as an "anti-atherosclerotic" agent, no information is as yet available about its direct influence on arterial tissue cells.

The present findings on the lipoprotein metabolism of cultured aortic SMC indicate that 5 mM of CPIB cause an enhanced retention of lipid-radioactivity ingested in the form of ^{125}I-lipoprotein by the cells from the culture medium. This effect is detectable even at concentrations which correspond to serum levels under CPIB therapy.

Since in a dynamic steady state of lipoprotein metabolism the observed ^{125}I-lipid content of aortic cells is equal to the sum of the uptake of ^{125}I-lipoprotein, their catabolism, and the elimination of noncatabolized ^{125}I-lipids, an increase of intracellular lipid radioactivity must result from an imbalance of these three processes. The data of Table I suggest that the CPIB induced lipid accumulation arises from a reduced elimination rather than from an enhanced entry of lipoproteins into the cells. This retention is considered to compensate for decreased cellular synthesis (see below).

The release of TCA precipitable radioactivity (containing both lipid and protein constituents) into the chase medium is a remarkable phenomenon indicating a limited ability of aortic SMC to catabolize ingested lipoproteins. The assumption that this is a special feature of cultured arterial wall cells is supported by similar observations of Bierman et al. (21) who found that aortic smooth muscle cells that had been pulsed for 24 hr in the presence of ^{125}I-HDL released about 50% of the label into the medium.

In the pulse chase experiments, a trypsinization of the SMC after pulsing was found necessary in order to release all the surface bound ^{125}I-lipoproteins which otherwise would be taken up in part or released into the medium during the subsequent chase. The possibility that the trypsinization promotes the release of intracellular material into the chase medium was excluded (see Results) and is also denied by Bierman et al. (21), who found no differences in the rate of release of HDL between nonreplated and (trypsin-treated) replated aortic smooth muscle cells.

The retention of lipoprotein-lipid radioactivity by aortic SMC may be interpreted as a consequence of the inhibitory effects of CPIB on the de novo fatty acid synthesis (Table IV), on the microsomal chain elongation (Table V), and on the formation of polyunsaturated longer chain fatty acids (8). The resulting deficiency in fatty acids required for the cellular lipid metabolism might be compensated for by an increased retention of ingested lipids which, after lysosomal degradation, provides the missing fatty acids. The fact that CPIB favors the transacylation from triglycerides to phospholipids supports this hypothesis (Table III).

The CPIB-induced retention of lipid-bound radioactivity does not imply an increase in the cellular lipid content. Although the proportion of ^{125}I-labeled lipid is higher in CPIB exposed cells, an equal rate of lipid synthesis in the control cells as well as in the treated cells would be possible under the assumption that the latter synthesize their lipids by utilizing the fatty acids of ingested lipoproteins. Incubation experiments made in the presence of [1-^{14}C]-acetate show that CPIB depresses overall synthesis of palmitic acid and causes a shift from de novo synthesis to chain elongation (Table IV).

In previous studies on bovine arterial cells (1), it was shown that a transition from de novo synthesis of fatty acids to chain elongation occurs when the ATP/ADP ratio decreases to a value of about 2. The assumption that CPIB suppresses the ATP formation possibly as a consequence of the inhibited pyruvate oxidation, is in accordance with the observation that CPIB reduces oxidative phosphorylation in rat liver mitochondria (26).

Cultured arterial smooth muscle cells express their characteristic phenotype even under culture conditions. This is indicated not only by their ability to bind, absorb and metabolize homologous lipoproteins but also by the overall glycosaminoglycan pattern of SMC (17) which closely resembles that of aortic tissue. Therefore, it seems likely that cultured aortic cells display a similar metabolic response to the action of CPIB as does the aortic tissue after the in vivo administration of CPIB. Our results, however, do not support the concept that CPIB has a significant effect in preventing lipid accumulation in arteriosclerosis.

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SHORT COMMUNICATIONS

A Simplified Colorimetric Micromethod for Determination of Serum Cholesterol

ABSTRACT

A simple and rapid micromethod is described for quantitation of cholesterol in 5-10 μl of serum using uranyl acetate to deproteinize the serum and *o*-phthalaldehyde reagent to develop color. Independent analyses of serum samples by the present method and by two reference methods, Abell-Kendall and automated colorimetric method, indicated accuracy of the micromethod (r = 0.99). Use of capillary blood specimens makes this procedure applicable for screening infants and small children.

INTRODUCTION

Since serum cholesterol level has assumed an increasingly important role in coronary artery disease, numerous methods for measuring cholesterol have been developed. The procedure of Abell et al. (1) is by far the most accurate and precise and is, therefore, considered the reference method for cholesterol determination. The need remains, however, for a simpler, less time-consuming, and accurate procedure.

Zlatkis and Zak (2) described a rapid, sensitive, and highly reproducible colorimetric procedure for measuring cholesterol by a new reagent, *o*-phthalaldehyde. Rudel and Morris (3) have successfully applied this method to biological samples; however, this procedure involves saponification, extraction, and evaporation steps prior to color development. Uranyl acetate, known to be an excellent fixative for proteins (4), was recently used by Jung et al. (5) in conjunction with ferric acetate to precipitate serum proteins. We adapted techniques to develop a micromethod of serum (plasma) cholesterol analysis which uses uranyl acetate to deproteinize the serum and *o*-phthalaldehyde reagent to measure cholesterol.

MATERIALS AND METHODS

Plasma/Serum

Serum or plasma was obtained by venipuncture and/or fingerstick from laboratory personnel, patients of Chairty Hospital in New Orleans, Louisiana, and from children who participated in the Bogalusa Heart Study of the LSU Specialized Center of Research–Arteriosclerosis. For fingerstick, we collected blood in 75-mm heparinated microhematocrit capillary

TABLE I

Cholesterol Analyses of Different Serum Samples[a]

Method	mg/100 ml Mean	SE	Range
Present procedure[b]			
1. n = 14	206	16.41	130-320
2. n = 34	177	6.96	132-290
Abell-Kendall[b]			
1. n = 14	210	17.26	137-329
LRC-CDC[c]			
1. n = 14	196	16.36	127-318
2. n = 34	172	6.45	134-276

[a]Samples were analyzed in two different batches, 1 and 2.

[b]Performed in SCOR-A Research Laboratory by a single analyst.

[c]Performed independently by different analysts in SCOR-A Core Lipid Laboratory.

TABLE II

Cholesterol Analyses on Capillary Blood vs. Venous Blood

Sample	mg/100 ml Capillary blood	Venous blood
1	143	143
2	185	185
3	176	178
4	244	239
5	141	141
6	232	224
7	158	157
8	140	143
9	133	133
10	216	215

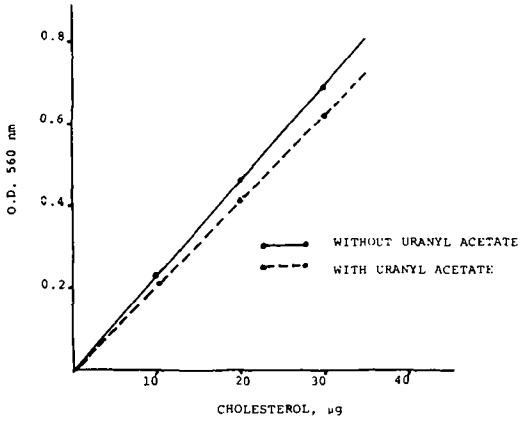

FIG. 1. Standard curve for cholesterol determination. Color was developed using *o*-phthalaldehyde reagent in the presence or absence of uranyl acetate.

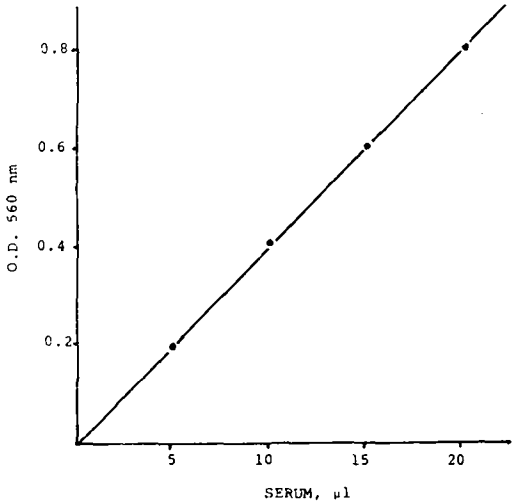

FIG. 2. Relationship between absorbance and serum volume. The absorbance obtained for 5 to 20 µl serum was equivalent to a serum cholesterol range of 95 to 386 mg/100 ml.

tubes after wiping off the interstitial fluid. One end of the tube was spun in a hematocrit centrifuge for 5 min. For cholesterol assay, aliquots of plasma (5-10 µl) were directly taken from the capillary tube with a microliter syringe and needle.

Reagents

o-Phthalaldehyde reagent (Sigma Chemical Co., St. Louis, MO) with a concentration of 1 mg/ml in glacial acetic acid was prepared fresh daily. Uranyl acetate (Baker Chemical Co., Phillipsburg, NJ) reagent (20 mg/100 ml), which is stable, was prepared in glacial acetic acid. Cholesterol standard solution (Eastman Kodak Co., Rochester, NY) consisted of 100 mg/100 ml glacial acetic acid.

Procedure

Aliquots of 5- to 10-µl of serum or plasma and 1.0 ml of uranyl acetate reagent were placed in microcentrifuge tubes (1.5 ml) and mixed thoroughly. After incubation for 10 min at room temperature, the tubes were centrifuged for 10 min. The clear supernatants were then transferred carefully to test tubes (13 x 100 mm). Similarly, a blank (0.15 M NaCl) and standards were run simultaneously. One ml of *o*-phthalaldehyde reagent was added to each tube, and the contents were thoroughly mixed before being allowed to stand for 10 min. Thereafter, 1.0 ml of concentrated sulfuric acid was carefully added by allowing it to run down the inside of the tube; the contents were immediately mixed with a vortex-type mixer and allowed to cool to room temperature. [Since the reaction mixture is viscous, mixing should be continued until no striations (schlieren) are visible.] Absorbance was read at 560 nm between 10 and 90 min after the addition of the sulfuric acid. For comparison, we also determined serum cholesterol by the Abell-Kendall method (1) and by a Technicon AutoAnalyzer II (Technicon Instrument Corp., Tarrytown, NY) according to the protocol developed by Lipid Research Clinics (LRC) and the U.S. Center for Disease Control (CDC) (6).

RESULTS AND DISCUSSION

Standard curves were constructed with and without uranyl acetate, as shown in Figure 1. Irrespective of the presence of uranyl acetate, there was a linear relationship between the degree of color development and the amount of cholesterol which was similar to that reported by earlier investigators (2,3). The presence of uranyl acetate caused about 8% reduction in absorbance per microgram of cholesterol standard. Since this reduction in absorbance was consistent over the entire range of observation, it should not affect the absolute cholesterol values of samples. Furthermore, the linear relationship shown in Figure 2 between the absorbance and increasing aliquots (5-20 µl) of serum indicates an effective deproteinization by uranyl acetate and complete recovery of serum cholesterol in the supernatant.

To determine the accuracy of the method, serum samples were analyzed in two different batches independently by the present method (in duplicate) and by the reference Abell-Kendall method and/or by the automated LRC-CDC cholesterol method. Although this

method does not involve hydrolysis of cholesteryl esters, the results, shown in Table I, indicate a good agreement between the methods (correlation coefficient 0.99). As in other methods (6), it is likely that marked differences in cholesteryl ester composition in some occasional samples may influence the results. Duplicate analyses by the present method gave percent mean (range) variations of ± 1.2 (0-6.9) for the first batch and ± 0.81 (0-6.7) for the second batch. Since this method can determine as little as 5 μg of cholesterol, the feasibility of measuring cholesterol on capillary blood samples was studied, and the results are compared with the analyses on venous blood samples of the same individuals. Plasma cholesterol levels, shown in Table II, indicate very good agreement between the capillary and venous blood samples. There was no interference in the color development by either bilirubin at 1-10 mg/100 ml levels or by hemoglobin at concentrations of 0.1-0.4 g/100 ml. Uranyl acetate is known to reduce interference by noncholesterol chromogens and eliminate intereference by bilirubin (5).

Thus, the cholesterol assay system described above compared favorably with the reference method and offers several advantages. Elimination of solvent extraction and saponification steps and use of capillary blood specimens should make this procedure suitable for screening infants and small children, either in doctors' offices or in well-baby clinics. Also, the use of capillary blood would facilitate repetitive sampling for clinical and experimental studies and allow inexpensive population screening.

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Fatty Acid and Sterol Synthesis by Rat Small Intestine in vitro

ABSTRACT

Slices of rat jejunum were incubated with [2-^{14}C]pyruvate, [1-^{14}C]acetate, or [^{3}H]H_2O to determine lipogenic activity. Under all conditions studied, pyruvate acted as a better precursor than acetate for fatty acid synthesis but not for the synthesis of sterol. Exogenous glucose significantly ($P \leq 0.05$) increased the conversion of both pyruvate and acetate to fatty acids. By contrast, fasting resulted in a decrease ($p \leq 0.05$) in lipogenic activity. The highest levels of lipogenesis were observed when [^{3}H]H_2O + glucose at a concentration of 20 mM was used. From such experiments, the absolute rate of fatty acid synthesis in the tissue preparation was calculated: 734 ± 54 nmoles acetyl units incorporated into fatty acids/g tissue/hr.

INTRODUCTION

Previously reported values for the conversion of [^{14}C]acetate to fatty acids by preparations of rat small intestine range from a low of 60 nmoles (1) to a high of 200 nmoles (2) acetyl units converted to fatty acids/g tissue/hr. Those for cholesterol synthesis range from 2 nmoles (3) to more than 60 nmoles (4,5) acetyl units converted to cholesterol/g tissue/hr. Such

method does not involve hydrolysis of cholesteryl esters, the results, shown in Table I, indicate a good agreement between the methods (correlation coefficient 0.99). As in other methods (6), it is likely that marked differences in cholesteryl ester composition in some occasional samples may influence the results. Duplicate analyses by the present method gave percent mean (range) variations of ± 1.2 (0-6.9) for the first batch and ± 0.81 (0-6.7) for the second batch. Since this method can determine as little as 5 μg of cholesterol, the feasibility of measuring cholesterol on capillary blood samples was studied, and the results are compared with the analyses on venous blood samples of the same individuals. Plasma cholesterol levels, shown in Table II, indicate very good agreement between the capillary and venous blood samples. There was no interference in the color development by either bilirubin at 1-10 mg/100 ml levels or by hemoglobin at concentrations of 0.1-0.4 g/100 ml. Uranyl acetate is known to reduce interference by noncholesterol chromogens and eliminate intereference by bilirubin (5).

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TABLE I

Conversion of Pyruvate or Acetate to CO_2, Fatty Acids, and Sterol by Slices from Intestines of ad libitum Fed and Fasted Rats[a]

Dietary	Substrate	(nmoles substrate converted/g tissue/hr) CO_2	Fatty acids	Sterol
Ad libitum (5)	Pyruvate + Glucose	7487 ± 586[b]	673 ± 91[b]	74 ± 14[c]
	Acetate + Glucose	4627 ± 106	305 ± 27	68 ± 7
Ad libitum (5)	Pyruvate	9271 ± 568[b]	408 ± 80[b]	63 ± 12[b]
	Acetate	6513 ± 118	183 ± 16	30 ± 5
Fasted (7)	Pyruvate	9946 ± 999[b]	185 ± 20[b]	24 ± 4[c]
	Acetate	6627 ± 629	121 ± 13	28 ± 6

[a]One hundred milligrams of tissue slices were incubated with 20 μmol of [2-^{14}C] pyruvate (0.25 μCi) or 20 μmol [1-^{14}C]acetate (0.25 μCi) in 2.0 ml Krebs-Henseleit bicarbonate buffer, pH 7.3 at 37 C in an atmosphere of 95% O_2/5% CO_2 for 2 hr. Where indicated, glucose was added at a concentration of 10 mM. Results are presented as means ± SE with the number of animals presented in parentheses; b = $p \leq 0.05$; c = $p \geq 0.05$.

differences reflect in part differences in experimental conditions, and with respect to cholesterol synthesis, the portion of the small intestine used (2,4,5). However, such discrepancies could also indicate that acetate may not be the best precursor with which to measure intestinal lipogenesis. In an attempt to resolve the discrepancies, therefore, this study compared the conversion to lipid of [1-^{14}C] acetate to that of [2-^{14}C] pyruvate by slices prepared from rat jejunum. To investigate the possible effects of dietary manipulation on intestinal lipogenesis, one group of animals was fasted for 24 hr. Finally, to establish an estimate of the absolute rate of fatty acid synthesis in the tissue preparation, [^{3}H] H_2O was used.

EXPERIMENTAL PROCEDURES

Male Long-Evans rats weighing 250-300 g were used. Animals were housed under standard conditions of temperature (24 C) and lighting (0600-1800 light; 1800-0600 dark) and were allowed water and stock ratio (Wayne Lab Blox) ad libitum. Fasted animals were without food for 24 hr prior to sacrifice.

Animals were sacrificed by cervical dislocation and allowed to bleed freely from carotid arteries. Immediately thereafter, the entire small intestine from pylorus to cecum was excised and flushed with copious ice-cold 0.9% NaCl. A 10-12 cm length was cut from the intestine immediately distal to the duodenum. The segment so obtained was placed on a cold glass plate, opened longitudinally along the mesenteric border leaving the mucosal side up, and again rinsed with ice-cold 0.9% NaCl. Then, with a razor blade, 3 mm thick slices were prepared freehand and transferred to cold Krebs-Henseleit bicarbonate buffer which had been previously equilibrated at pH 7.3. Precisely weighed portions of intestinal slices were incubated under the conditions detailed in the legends for the tables. Methods for determining the conversion of labeled substrates to fatty acids, nonsaponifiable lipid, and CO_2 were analagous to those previously described in detail for liver slice experiments (6). Throughout this paper, the term "sterol" refers to the nonsaponifiable lipid fraction.

TABLE II

Effect of Glucose Concentration on Incorporation of ^{3}H from [^{3}H]H_2O into Fatty Acids and Sterol[a]

Glucose (mM)	(ng atoms ^{3}H incorporated/g tissue/hr) Fatty acids	Sterol
0	378	114
10	1036	249
20	1287 ± 95 (4)	350 ± 87 (4)
50	1390	302

[a]Two hundred milligrams of tissue slices were incubated with [^{3}H]H_2O (0.5 mCi) and glucose at the indicated concentrations in 2.0 ml Krebs-Henseleit bicarbonate buffer, pH 7.3 at 37 C in an atmosphere of 95% O_2/5% CO_2 for 2 hr. Results are presented as means of closely agreeing duplicate determinations, or as means ± SE with the number of animals given in parentheses.

RESULTS AND DISCUSSION

Preliminary intestinal slice experiments indicated that the conversion of labeled substrate to lipids or CO_2 was approximately linear for at least 2 hr. All subsequent experiments involved a 2 hr incubation period.

Previous reports of rat intestinal slice experiments have shown that labeled acetate either with (1,5,7) or without (2-4) added glucose will support fatty acid (1,2,4,7) or sterol (1-5,7)

synthesis. The present study demonstrated that pyruvate acted as a better precursor than acetate for fatty acid synthesis but not for the synthesis of sterols (Table I). Furthermore, added glucose, although not required for lipogenesis, significantly ($p \leqslant 0.05$) [Student's *t*-test (8)] increased the conversion of both pyruvate and acetate to lipid (Table I) when added to a final concentration of 10 mM. These results were not in complete agreement with those of Franks et al. (1), who reported that optimum rates of conversion of [^{14}C]acetate by rat intestinal slices were obtained with glucose concentrations near 100 mg%.

In order to quantitate the absolute rate of lipogenesis in the tissue preparation, a series of experiments was undertaken with [^{3}H]H_2O (Table II). In agreement with the results obtained with [^{14}C] labeled substrates, exogenous glucose increased lipogenesis. Moreover, when the glucose concentration was varied over the range of 10-50 mM, maximum lipogenic activity was observed at the 20 mM concentration (Table II), and from these results, the absolute rate of fatty acid synthesis was calculated to be 734 ± 54 nmoles actyl units converted to fatty acids/g tissue/hr (9). That rate was not significantly greater ($p = 0.06$) [Student's *t*-test (8)] than that observed with [^{14}C] pyruvate (Table I).

The decrease in [^{14}C]CO_2 production due to glucose (Table I) was probably the result of dilution of the [^{14}C]acetyl CoA pool derived from [^{14}C]pyruvate or [^{14}C]acetate by unlabeled acetyl CoA units produced from added glucose. In this regard, it is noteworthy that the total extent of the increase in lipogenic activity due to glucose was much higher than that observed (Table I) but cannot be estimated because of substrate dilution.

When the possible effects of fasting were investigated, lipogenesis was significantly ($p \leqslant 0.05$) [Student's *t*-test (8)] decreased (Table I). A decrease in human intestinal lipogenesis due to fasting has been previously reported (10). The changes we observed due to fasting were more pronounced when [^{14}C] pyruvate was used to measure lipogenesis than when [^{14}C]acetate was used, another indication that pyruvate provided a better estimate of lipogenic activity and the changes therein than did acetate.

Differences in the conversion rates of pyruvate and acetate to fatty acids and CO_2 can be attributed to differences in the pathways by which these substrates gain access to mitochondrial sites for fatty acid synthesis or oxidation. In this connection, Franks et al. (1) demonstrated that added carnitine increased by 50-70% the conversion of [^{14}C]acetate to lipid and its oxidation to CO_2 by rat jejunal slices. The present study (Table I) showed that under similar experimental conditions, slices of rat jejunum converted significantly ($p \leqslant 0.05$) more pyruvate to fatty acids and CO_2 than acetate. Acetate activation was not rate-limiting as indicated in experiments where added glucose increased the conversion of acetate to fatty acids (Table I). Thus, there appear at least two possibilities: either the movement of acetate into the mitochondria involves steps which are rate-limiting in the overall pathway for the conversion of acetate to fatty acids and its oxidation to CO_2, or the acetyl units produced from the oxidation of pyruvate and those obtained from acetate may find their way into two functionally distinct pools which do not equilibrate and which do not have equal access to mitochondrial pathways for fatty acid synthesis or oxidation to CO_2. In this regard, previous investigations with rat liver mitochondria (11) and lactating mouse mammary gland slices (12) have led others to suggest that acetyl CoA units from the oxidation of pyruvate and fatty acids may exist in two functionally distinct pools which do not equilibrate. Whatever the pathway(s) or substrate pool (s), the data in Table I indicate the presence of a substantial level of intramitochondrial fatty acid synthesis in rat small intestine. Investigations are now underway in this laboratory to quantitate and define the mode(s) of that synthesis.

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Hydrolysis of *bis*-Phosphatidic Acid from Developing Soybean by Phospholipase A_2

ABSTRACT

bis-Phosphatidic acid from developing soybean was completely hydrolyzed with phospholipase A_2 (lyophilized venom from Ophiophagus hannah) to yield lyso-bis-phosphatidic acid and free fatty acids. It is suggested that soybean bis-phosphatidic acid is derived from sn-3-glycerophospho-sn-3′-glycerol.

INTRODUCTION

bis-Phosphatidic acid (BPA), the fully acylated analog of phosphatidyl glycerol, was recently identified as a major lipid constituent of developing soybean (1). This phospholipid had previously been shown to have a relatively high rate of turnover with respect to its fatty acids in both soybean slices (2) and suspension cultures (3). bis-Phosphatidic acid was also found in cultured hamster fibroblasts (4); its stereoconfiguration was determined after strong alkaline hydrolysis (5), and most of it was found to be derived from sn-1-glycerophospho-sn-1′-glycerol.

So far, experiments on phospholipase hydrolysis of naturally occurring BPA have not been reported. Since phospholipase A_2 (phosphatide 2-acyl-hydrolase, EC 3.1.1.4) is known to be specific for phospholipids derived from sn-3-glycerophosphate (6), its possible action on BPA was of particular interest. We now report that BPA from developing soybean is completely hydrolyzed by snake venom phospholipase A_2 to yield free fatty acids and lyso-bis-phosphatidic acid.

EXPERIMENTAL PROCEDURES

BPA was isolated and purified from the total lipids of immature soybeans, harvested ca. 20 days after flowering, as described previously (2). Methanolysis and analyses by thin layer chromatography (TLC) and gas liquid chromatography (GLC) were done as described (2). Hydrolysis with 99% aqueous acetic acid (7) was carried out at 100 C for 10 min.

Hydrolysis with phospholipase A_2 was carried out at room temperature in a 10 ml volumetric flask. BPA (5 mg) was suspended in 5 ml diethyl ether. The mixture was stirred vigorously, flushed with nitrogen, and 0.1 ml of the snake venom solution (lyophilized venom from Ophiophagus hannah, Miami Serpentarium Laboratories, Miami, FL 33156; 5 mg in 1 ml Tris buffer, 0.1 M, pH 7.5, containing 0.4 mM $CaCl_2$) was added. The reaction was continued overnight. The diethyl ether was then removed under a stream of nitrogen, and 2 ml of methanol-chloroform-water (5:3:2, v/v/v) were added. The solution was transferred to a separatory funnel, and 2 ml of chloroform was added. The lower phase was removed, and the upper phase was reextracted twice with an equal volume of chloroform-methanol (85:15, v/v). The combined lower phases were dried under reduced pressure and the residue redissolved in chloroform.

RESULTS AND DISCUSSION

Soybean BPA was completely hydrolyzed with snake venom phospholipase A_2. Analysis of the reaction products by TLC on Silica Gel H (Merck) using chloroform-methanol-ammonia (65:20:4, v/v/v) showed the absence of BPA (R_f 0.53) and the presence of only one phosphorus positive spot (R_f 0.24); another fraction (R_f 0.32) corresponded to free fatty acids. Both the free fatty acids and the lysophospholipid were isolated by preparative TLC using diethyl ether-acetic acid (99:1, v/v) and were reacted with MeOH/HCl. The fatty acid methyl esters were analyzed by GLC (EGSS-X) and their absolute amount estimated by GLC

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TABLE I

Fatty Acid Composition (wt %) of bis-Phosphatidic Acid (BPA), Lyso BPA and Free Fatty Acids (FFA) Derived by Phospholipase A_2 Hydrolysis, and Their Calculated Average (Av)

Fatty acid	BPA	Lyso BPA	FFA	Av
16:0	18.6	31.4	5.6	18.5
16:1	1.0	--	2.3	1.2
18:0	8.5	20.4	2.0	11.2
18:1	6.3	7.0	5.2	6.1
18:2	41.8	28.6	52.3	40.4
18:3	23.8	12.6	32.6	22.6

(OV-1) after adding an internal standard of methyl heptadecanoate.

Approximately equal amounts of fatty acid methyl esters were obtained from the free fatty acid (48.6%) and the lysophospholipid (51.4%) fraction; their compositions are listed in Table 1.

The structure of the lysophospholipid was established as lyso BPA by hydrolysis with acetic acid (4,7), which produced monoacylglycerol and lysophosphatidic acid, as determined by TLC using hexane-diethyl ether (1:1, v/v) and chloroform-methanol-water (65:25:4, v/v/v). Thus, phospholipase A_2 completely hydrolyzed the acyl groups at positions 2 and 2′ of soybean BPA.

Although an unequivocal determination of the absolute configuration of soybean BPA will have to depend on the chemical synthesis and analysis of all possible stereomers, our present results strongly suggest that it is derived from sn-3-glycerophospho-sn-3′-glycerol. This view is supported by the finding that a racemic mixture of BPA (Serdary Research Laboratories, London, Ontario) yielded only the expected one-fourth of its total phosphorus in lyso-bis-phosphatidic acid after phospholipase A_2 hydrolysis, whereas synthetic BPA, prepared (8) from 1,2-diacyl-sn-glycerol (Serdary), was completely hydrolyzed.

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ERRATUM

In "Lipid Metabolism of the Yellow Clam, *Mesodesma mactroides:* 3-Saturated Fatty Acids and Acetate Metabolism" [*Lipids* 12:804 (1977)], the title was printed incorrectly. The corrected title should read, "Lipid Metabolism of the Yellow Clam, *Mesodesma mactroides:* III. Saturated Fatty Acids and Acetate Metabolism."

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